An Unexpectedly Large Polyclonal Repertoire of HPV-Specific T Cells Is 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 patients with cervical cancer, by systematic analyses of the proportion, breadth, and polarization of human papillomavirus (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 usage. HPV-specific CD4+ and CD8+ T-cell responses were detected in TIL and TDLNC and their relative contribution varied between <1% and 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 T-cell receptor Vβs 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.

Cancer Res; 70(7); 2707-17. ©2010 AACR.

Introduction

Our current knowledge on the diversity and extent of tumor-specific T-cell immunity is largely based on pooled T-cell response data from 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 an individual's immune system could simultaneously respond to one or more tumor antigens. Cervical carcinoma offers an excellent opportunity to answer this question as it is 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 antitumor 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 HPV18, the TILs did not react to these HPV16 or HPV18 peptides (3). Although these studies clearly indicate that HPV-specific T cells could 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

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-09-4299
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be influenced by the presence of a preexisting HPV-specific local immune response.

Therefore, we performed a more in-depth study of the popu-
lations of TIL and TDLNC in patients, which previously
were shown to comprise HPV16- or HPV18-specific T cells (3)
using a 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.

Materials and Methods

Subjects. Women presenting with histologically proven cer-
vical neoplasia (International Federation of Gynecology and
Obstetrics 1a2, 1b1/2) at the Department of Gynecology of
the Leiden University Medical Centre were enrolled in the CIR-
CLE study, which investigates cellular immunity against cer-
vical 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 resi-
dues spanning both HPV16 and HPV18 E6 and E7 proteins
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 de-
scribed previously (3). Briefly, fresh cervical tissue was min-
ced and cultured in Iscove’s modified Dulbecco’s medium,
supplemented with 10% human AB serum (PAA Labora-
tories), 10% T-Cell Growth Factor (Zapetemrix), and 5 ng/
mL of interleukin-15 (IL-15; Peprotech). On day 1, 5 ng/mL
of IL-7 (Peprotech) was added to cultures to drive the ho-
meostatic expansion of T cells. This does not alter the
CD4/CD8 T-cell composition of TIL (3), but it allows the
acquisition of a sufficient number of T cells for immunologic
assays. After 2 to 3 wk of T-cell expansion (mean 18 × 10⁶
cells, range 4 × 10⁶ to 40 × 10⁶), 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 in-
to pieces and incubated for 1 h at 37°C with collagenase (200
IU/mL; Sigma) and DNase (50 μg/mL; Sigma), and put
through a cell strainer (BD) 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 EBV transformed B cell
lines (B-LCL) pulsed with HPV16 or HPV18 E6 and E7 pep-
tides (5 μg/mL) in triplicate wells in a 3-d proliferation assay.
After 48 h, supernatant was harvested and stored at −20°C for
cytokine analysis. During the last 16 h of culture, 0.5 μCi/well
of [3H]thymidine was added to measure proliferation (27).
The stimulation index was calculated as the average of test
wells divided by the average of the medium control wells. A
stimulation index of >2 was considered a positive response.

Antigen-specific IFNy and IL-10 production was measured
by ELISA (29). Antigen-specific cytokine production was de-
fined by a cytokine concentration above the cutoff value
(IFNy, 100 pg/mL; IL-10, 20 pg/mL) and more than two times
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 μg/mL of
HPV16 or HPV18 E6 and E7 peptide pools. TILs or TDLNC
were thawed, rested in Iscove’s modified Dulbecco’s medium
(Bio Whittaker) containing 10% FCS (FCS, PAA Laborato-
ries) for 5 h and seeded into a 96-well round-bottomed plate at
200,000 cells per well and 40,000 antigen-pulsed B-LCL were
added. After 1 h, Brefeldin A (10 μg/mL) was added to the
culture and left overnight. Cells were stained with anti-
bodies to CD154-PECy5, CD137-APC, CD3-Pacific Blue, CD4-
PECy7, CD8-APCcy7, IFNy-FITC, and IL-2-PE (all from BD
PharMingen; ref. 30).

In addition, TDLNC and TIL were stimulated with a mix of
irradiated autologous B-LCL and 5 μg/mL of HPV16 or
HPV18 E6 or E7 peptide pools and irradiated allogeneic pe-
ripheral blood mononuclear cell pool to obtain enough HPV-
specific T cells to measure the breadth of the response with
respect to single peptide-antigens and for T-cell receptor Vβ
(TCRVβ) usage analyses (eight sets of antibodies). After a 3-
wk 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 re-
ponse. In vitro expanded T cells were stimulated with the
indicated single peptides of HPV16 or HPV18 E6 and E7
(5 μg/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). A TCRVβ was con-
didered dominant (>10%), subdominant (3–10%), or minor
(<3%) on the basis of the percentage of HPV-specific cells us-
ing the same TCRVβ.

Reverse transcription-PCR. Expanded cultures were en-
riched for CD8+ T cells by negative selection using CD4+ iso-
lation dynal beads (Invitrogen). After RNA was isolated with
the RNeasy mini isolation kit (Qiagen), cDNA was synthe-
sized using the iScript cDNA Synthesis kit (Bio-Rad). 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 Toll-like receptor
ligands. TDLNC were thawed, rested for 5 h and stimulated
with a 1 μg/mL pool of E6 and E7 peptides, T-Cell Growth
Factor 10%, and IL-15. Toll-like receptor 4 (TLR4) ligand
lipopolysaccharide (250 ng/mL; Sigma-Aldrich), TLR3 ligand
Poly(I:C) (12.5 μg/mL; InvivoGen), and TLR1-2 ligand
Pam3CSK4 (20 μg/mL; InvivoGen) were added at the start
of the culture where indicated. To stimulate TIL, monocytes
were pulsed with 1 μg/mL of the E6 and E7 peptide pool and
the indicated TLR ligands. After 5 h, monocytes were washed and TIL were added. Supernatant was taken every 2 d and analyzed by human Th1/Th2 cytometric bead array (BD PharMingen). Cells were left to rest for 2 to 3 wk before the 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 with TIL (8 patients) or TDLNC (2 patients) which contained T cells that specifically reacted to the peptides of the HPV type present in the tumor, 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 of 12 tumors tested. All HPV-specific T-cell cultures produced IFNγ, yet the amount of production varied greatly (103 to >5,000 pg/mL), irrespective of the proliferative capacity of the cells. In addition, four of eight positive cultures produced IL-10 (45–836 pg/mL). All TIL reacted to phytohaemagglutinin and proliferation was associated with large amounts of IFNγ and IL-10 (data 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, Table 1.

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

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<th>Patient</th>
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<th>Overnight activation analysis</th>
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<td>LN</td>
<td>0</td>
<td>NT</td>
<td>—</td>
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</tbody>
</table>

*HPV type found in the tumor by PCR.
1Days of cytokine-mediated homeostatic expansion before immune assay.
2Percentage 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.
3Reactivity indicates the HPV type and protein to which the T-cell culture specifically reacted.
4SI, stimulation index, average proliferation of test wells divided by the average proliferation of medium control wells. SI > 2 is positive.
5Antigen-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).
6NT, not tested.
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 because 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 (Fig. 1B; Supplementary Fig. S1). The three TIL cultures which previously tested negative remained negative excluding priming in vitro. In most of the patients, the HPV-specific CD4+ T-cell response was highly diverse, as CD4+ T cells responded to five or more different peptides in 5 of 10 patients, and in another 4 patients, the CD4+ T cells recognized two different peptides (Supplementary Table S1). HPV-specific CD8+ T-cell reactivity was detected in 6 of the 12 tested patients, 5 of whom displayed CD8+ T-cell reactivity to two to six different peptides (Fig. 1B; Supplementary Table S1).

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 of 12) recognized three or more different T-cell epitopes (Fig. 1C). The TDLNC populations reacted against four to nine different epitopes (Fig. 1C). Thus, the tumor-induced HPV-specific T-cell repertoire is directed against multiple T-cell epitopes. Based on the patient’s HLA-type, four of the CD4 responses and two of the CD8 responses could involve a reaction against known HLA class I and II T-cell epitopes (data not shown; refs. 3, 34, 35). As each individual T-cell epitope could 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 TCRVβs were found (Table 2; Fig. 2B), as well as several subdominant and minor TCRVβs. For example, the HPV-specific CD4+ T-cell response of patient P14 reacted to nine different peptides and—on the basis of the different TCRVβs present in the population of activated T cells—this involved the activation of at least 43 different T-cell clones (Table 2; Fig. 2B). In a number of cases, not all TCRVβs could be identified because the available antibodies cover ~70% to 80% of the full TCRVβ repertoire (36). In one case (P7), in which 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 (Fig. 2A, fourth row; Table 2)—a semiquantitative reverse transcription-PCR (RT-PCR) was applied, revealing the presence of TCRVβ24 (Fig. 2C). In another case (P15), only 30% of the TCRVβs were accounted for by antibodies. Here, three additional TCRVβ 6C, 6D, and 15 (data 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 (Fig. 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 antitumor 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 (Fig. 3A and B). Profile 1 was found in one patient (P7). Despite the presence of a high percentage of HPV E6–specific CD8+ T cells (24%), only ~4% produced either one of the cytokines. The second profile comprised HPV-specific T cells in 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 4 (P14 and P9). These data indicate that whereas most of the HPV-specific TIL can produce either one of the type 1 cytokines, there are only few patients (two of nine) in whom the majority of their HPV-specific TIL simultaneously produce IFNγ and IL-2 (profile 2; Fig. 3A and B).

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 a strong production of IFNγ (Table 1; Fig. 3A and B). In mouse models, the local injection of TLR2, TLR3, or TLR4 ligands could augment the tumor response (43–45). To mimic the local delivery of antigen and TLR ligands, homeostatic expanded TIL from two patients were stimulated with HPV antigen-pulsed TLR-activated autologous monocytes whereas TDLNC from two other patients, which already contained antigen-presenting cells, 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 three out of four of the cultures with poly(I:C) (TLR3; Fig. 3C). The use of the TLR4 agonist lipopolysaccharide 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 three of four cultures (data not shown). Analysis of the constitution of the responding cell population after 14 days, giving the activated T cells some rest

![Image](https://example.com/image.png)

**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 columns) after antigen-driven expansion. The minimal number of epitopes recognized (black columns) was estimated by counting the response to two 22-mer peptides, which overlap by 12 amino acids, as one.
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 (data not shown).

Discussion

We have comprehensively analyzed the spontaneous tumor-specific immune response in patients with cervical cancer by dissecting local HPV E6- and E7-specific CD+ 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—indepedent 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 >40% to 50% of all patients with an HPV16- or HPV18-positive cervical carcinoma (3). Our data shows that whereas HPV-specific T-cell responses could 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 antitumor response varied enormously, ranging from <1% to 66% (Table 1). Although we cannot exclude that the quantification of HPV-specific T cells within the total population of TILs is accurate as it is 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 TCRβ families, or T-cell responses to multiple different epitopes within a single peptide (Table 2; Fig. 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 might not equally amplify all possible responding cells and less well-proliferating HPV-specific T-cell clones may even become extinct. However, in view of the broad responses previously observed, 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 cytomegalovirus (36). This brings forward the question of whether the HPV-specific T-cell responses observed in these cancer patients reflect a characteristic antiviral response or an antitumor response. As shown previously, patients with HPV-induced premalignant disease either fail to mount HPV-specific immunity (4, 12) or induce a nonbeneficial HPV-specific T-cell response during the 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 peptides–specific CD8 T-cell response (6). The presence of single peptide–specific dominant and subdominant (based on TCRβ chain expression) T cells within the tumor tissue implies that subdominant TIL participate in the immune surveillance of tumors and do 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 patients with cervical cancer studied previously (47). Overall, the local HPV-specific immune response consists of a polyclonal T-cell population that is able to respond to many different CD4 and CD8 T-cell epitopes. We deem it unlikely that this breadth is underestimated due to the potential cross-reactivity of the T cells with nonrelated 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; Fig. 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

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*Depicted are all patients of whom enough T cells were available for TCRVβ 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 TCRVβ consists of >10% of the activated T cells. A subdominant TCRVβ consists of between 3% and 10% of the activated T cells. A minor TCRVβ consists of <3% of the activated T cells.
§Additional TCRVβs were found by PCR analysis for P7 and P15.
implying that the local tumor-specific immune response in cervical cancer patients does not differ from others, such as in 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 Fig. 1A and B) as well as in an increased IFN-γ 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 (Fig. 3C). This suggests that local delivery of these innate immune-derived stimulating factors could stimulate a stronger antitumor 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 responses are highly required as they correlate with clinical efficacy in murine models (43) and in human trials (50). Interestingly, the widely used TLR4 ligand lipopolysaccharide did not overly increase IFNγ production of TIL and TDLNC in our study.

In conclusion, TIL or TDLNC isolated 40% to 50% of patients with an HPV16- or HPV18-induced cervical tumor containing HPV type–specific T cells (3). Our study of the local T-cell repertoire within this group of patients suggests that

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**Figure 3.** Functionality of HPV-specific TIL and TDLNC. A, the specific activity to HPV16 or HPV18 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); profile 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 columns. The stacked columns indicate the percentage and type of cytokines [black, IFNγ; gray, IL-2; hatched, IFNγ + IL-2; white, no IFNγ or IL-2 (none)] produced within the activated HPV-specific T-cell population. C, the homeostatic expanded TIL of patients 7 and 9 as well as the TDLNC of patients 13 and 14 were stimulated with HPV16/HPV18 E6 or E7 peptide pools and TLR-agonist when indicated (medium control indicates peptide stimulation without the addition of TLR agonist). After 2, 4, and 7 d, supernatant was harvested and analyzed for cytokine production by cytokine bead array.
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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

44. Currie AJ, van der Most RG, Broomfield SA, Prosser AC, Tovey MG, Robinson BW. Targeting the effector site with IFN-αβ-inducing TLR ligands reactivates tumor-resident CD8 T cell responses to eradicate established solid tumors. J Immunol 2008;180:1535–44.
An Unexpectedly Large Polyclonal Repertoire of HPV-Specific T Cells Is Poised for Action in Patients with Cervical Cancer

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*Cancer Res* 2010;70:2707-2717. Published OnlineFirst March 16, 2010.

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