Regulation and subversion of HPV16-specific immunity in cancer patients

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Chapter 4.1

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Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens

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Abstract. Because of their important role in the maintenance of self-tolerance, CD4+ regulatory T cells prevent autoimmune diseases but also curtail the efficacy of T-cell immune responses against cancers. We now show that this suppressive action of CD4+ regulatory T cells is not limited to cancers displaying tumor-associated self antigens, such as melanomas, but also extends to human papillomavirus (HPV)-positive cervical cancers that express foreign tumor antigens. HPV-specific CD4+ T cells isolated from lymph node biopsies of cervical cancer patients were found to suppress proliferation and cytokine (IFN-γ, IL-2) production by responder T cells. The capacity of HPV-specific CD4+ T cells to exert this suppressive effect depended on their activation by cognate HPV antigen and on close-range interactions with responder T cells. HPV-specific CD4+ regulatory T cells were also retrieved from cervical cancer biopsies, suggesting that they interfere with the anti-tumor immune response at both the induction and effector levels. Our findings offer a plausible explanation for the observed failure of the tumor-specific immune response in patients with cervical carcinoma.

Introduction

Cervical carcinomas arise as result of an uncontrolled persistent infection with a high-risk type of human papillomavirus (HPV), in particular, types 16 (HPV16) and 18 (HPV18), which account for approximately two-thirds of these cancers (1, 2). The HPV E6 and E7 proteins play a pivotal role in carcinogenesis and are expressed in both premalignant and advanced cervical lesions (3). Because HPV proteins are foreign to the body, one would expect the immune system to mount a response against these antigens when expressed in the cervical epithelium. Indeed, HPV16 E6-, E7-, and E2-specific Th1- and Th2-type CD4+ T cell responses were frequently detected in peripheral blood mononuclear cell (PBMC) cultures of healthy individuals (4-6), showing that successful defense against HPV16 infection is commonly associated with the installment of a systemic effector T-cell response against these viral antigens. In contrast, PBMC cultures from patients with HPV16-positive genital lesions either lacked detectable responses against HPV16 E6, E7, and E2 or displayed antigen-specific proliferative responses exhibiting a noninflammatory cytokine profile (5, 7, 8). Similarly, effective HPV18-specific T-cell responses are only found in healthy controls but not in HPV18-positive patients (9). Taken together, these findings indicate that the development of high-risk HPV-positive cervical cancer is associated with failure of the HPV-specific T-cell response.

Studies in mouse models demonstrated that CD4+ regulatory T cells play a critical role in curtailing effective immune responses against tumors (10, 11) and that these T cells can be primed in the same tumor-draining lymph nodes as their tumoricidal counterparts (12). Furthermore, analysis of tumor biopsies from melanoma patients revealed the presence of CD4+ regulatory T cells recognizing the cancer-testis autoantigen LAGE-1 (13, 14). The failure of the HPV-specific immune defense in patients with cervical neoplasia prompted us to investigate whether the conditions at the interface of lesion and immune system would likewise promote the induction of antigen-specific CD4+ regulatory T cells against the foreign HPV16/18 E6 and E7 oncoproteins.

This report demonstrates that HPV-specific CD4+ regulatory T cells can be isolated from lymph node biopsies of cervical cancer patients and, moreover, that such T cells can infiltrate tumors. These data indicate that antitumor immunity in cervical cancer patients is suppressed at both the induction and effector levels. The presence of HPV-specific CD4+ regulatory T cells may not only impact the natural T-cell immune surveillance but also T-cell responses induced by therapeutic anticancer vaccines.

Materials and Methods

Patients. Women with histologically proven HPV16- or HPV18-positive (15) cervical carcinoma were enrolled after providing informed consent. The study design was approved by the Medical Ethical Committee of the Leiden University Medical Centre.

T Cells and APCs. Tumor-positive lymph nodes were minced and digested with collagenase (200 units/ml; Sigma, Bornem, Belgium) and DNase (50 μg/ml; Sigma) for 1 h at 37°C and dispersed through a cell strainer (BD, Erebodemgem, Belgium). LNMC were stimulated with either a pool of all E6 or E7 peptides at 2.5 μg/ml in Iscove's modified Dulbecco's medium containing 10% human Ab serum (Sigma), 10% T cell growth factor (Zeptometrix, Buffalo, NY), and 5 ng/ml IL-15 (Peprotech, Rocky Hill, NJ). T cell clones were isolated by limiting dilution (16) in which IL-2 was replaced by 10% T cell growth factor, 5 ng/ml IL-15, and 0.5 μg/ml phytohemagglutinin (Murex Diagnostics, Dartford, U.K.). A set of overlapping peptides for E6 and E7 of HPV16 and HPV18 (6, 9) and recombinant E6 and E7 proteins of HPV 16 and 18 (17) were used as antigens in T cell stimulation assays. T cell clones C148, C151, and C271 recognized peptides 16E6 11-32, 18E6 51-72, and 16E6 11-32, respectively, in a HLA class II-restricted fashion (SI Figure 8). Growing T cell clones could be maintained for months by culture in Iscove's modified Dulbecco's medium containing 10% FCS (PAA Laboratories, Pasching, Austria), 10% T cell growth factor, and 5 ng/ml IL-15 and expanded using a mix of irradiated PBMCs from three different donors, EBV B-LCL, and 0.5 μg/ml phytohemagglutin. HPV-specific T cell lines were established from minced tumor biopsies (unpublished data) and from HPV16 E6-positive skin test biopsies from two different healthy subjects who participated in a HPV16 peptide delayed-type hypersensitivity pilot study (unpublished data). The HPV-specific T cell lines recognize 16E6 129-138 (176), 16E6 37-68 (226), 16E6 41-62 (265), 16E6 91-112 (331), 16E6 109-158 (P02), and 16E6 55-104(P08). The healthy subject-derived HPV16 E6 73-104-specific Th1 clone 24.101 was isolated from a rapidly enriched PBMC culture by limiting dilution (18).

The influenza-specific T-helper 1 (Th1) clone 3 was established by repeated stimulation of LNMC with a pool of influenza matrix 1 (M1) peptides (17), followed by limiting dilution. This clone recognizes the influenza M1 peptide VTTTNPLIRHENRMVLASTTAKAMEQMA and produces high levels of IFN-γ, TNF-α, and IL-2. The HIV-specific Th1 clone 36 recognizes the reverse transcriptase peptide FRKQNPDIVIYQYMDDLYVG and produces high levels of IFN-γ and IL-2 (19).

A standardized mixture of five EBV B-LCLs, including B-LCLs from patients C148, C151, and C271 and two different B-LCLs of choice (depending on responder cells), which together expressed all relevant HLA class II molecules, served as a standardized APC population for all CD4+ T-cell subsets used in our experiments.

Proliferation Assays, Cytokine ELISA, and Suppression Assays. Antigen-specific proliferation was measured in standard assays (17). IFN-γ and IL-10 (Sanquin, Amsterdam, The Netherlands) and IL-2 (Diaclone,

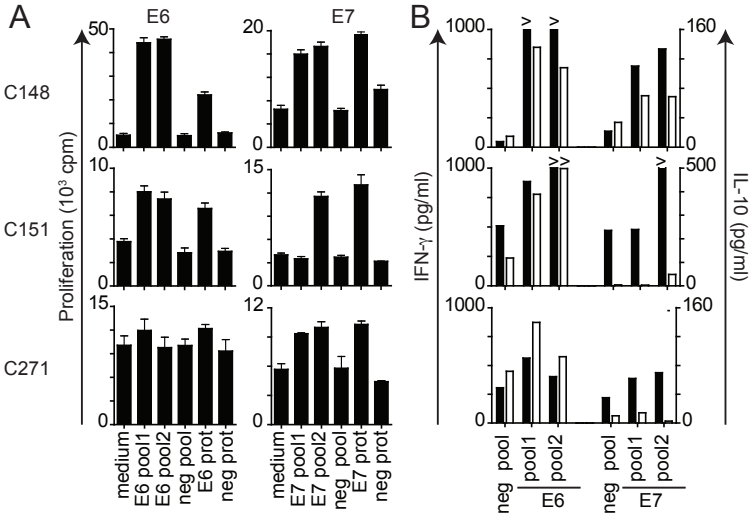

Figure 1. HPV-specific T cells are present in tumor-draining lymph nodes. A) The HPV-specific proliferative response of LNMCs from patients with HPV16+ (C148 and C271) and HPV18+ (C151) cervical carcinoma was analyzed in a [^{3}H]thymidine incorporation assay. A Left shows LNMC cultures that were tested for their reactivity against pools of E6 peptides (HPV16 E6 pool 1: aa 1–86, pool 2: aa 73–158; HPV18 E6 pool 1: aa 1–92, pool 2: aa 71–158) or the corresponding recombinant protein. A Right depicts cultures that were tested against pools of E7 peptides (HPV16 E7 pool 1: aa 1–56, pool 2: aa 43–98; HPV18 E7 pool 1: aa 1–62, pool 2: aa 41–106) or the corresponding recombinant protein. In the case of cultures obtained from HPV16+ patients, the peptide pools and recombinant protein of HPV18 were used as negative controls and vice versa. B) The IFN-γ (filled bars) and IL-10 (open bars) secreted by the T-cell cultures shown in A was measured in the culture supernatant by ELISA. Bars marked with > indicate that the amount of cytokine produced exceeded that of the standard.

Stamford, CT) levels were measured by using ELISA. The capacity to suppress the CD3 antibody (OKT-3; Ortho Biotech, Bridgewater, NJ) induced proliferation of naïve CD4+CD25− T cells, isolated by depletion of the CD25+ fraction using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by isolation of CD4+ lymphocytes using Dynal beads (Invitrogen, Breda, The Netherlands), was tested according to Levings et al. (20). T-cell suppression assays were only performed with T cell clones or lines that were rested for 2–5 weeks after their phytohemagglutin/antigen, IL-15, and T cell growth factor-induced expansion period. Transwell experiments were performed according to Wang et al. (13) using 1 μg/ml anti-CD3 antibody and 2 × 10^4 APCs in both inner and outer wells. Measurement of the suppressive capacity of HPV-specific T cells using CFSE-labeled (Invitrogen) responder cells and PKH26-labeled (Sigma) regulatory T cells was tested as described (21). In a number of experiments, HPV-specific T cells were treated with 50 μg/ml mitomycin C (Kyowa, Hakko, Japan) for 1 h followed by irradiation (2,000 rad) to prevent proliferation but not effector function of T-cell clones.

Suppression of IL-2 secretion was measured in the supernatant according to Wang et al. (13). Briefly, Th1 cells were labeled with CFSE, and 10^5 CFSE-labeled Th1 cells were cocultured with HPV-specific T cells at a 1:1 ratio together with 10^4 autologous EBV-transformed B cells for both clones and 50 units/ml IL-2. The HPV-specific T-cell clone was prestimulated with 10 μg/ml cognate peptide for 24 h (hereafter the Th1 clone was stimulated with 1 μg/ml cognate peptide), and 1 h later 10 μg/ml Brefeldin A (Sigma) was added. After overnight incubation, the cells were fixed, permeabilized, and stained with an APC-labeled CD4 antibody and intracellular IL-2-phycoerythrin (PE) (Becton Dickinson, Franklin Lakes, NJ). The percentage of IL-2-producing cells was analyzed by FACS.

FACS Analysis of Regulatory T-Cell Markers. HPV-specific T-cell clones were stained 3 weeks after their last antigen-specific activation in vitro with CTLA-4 PE (Becton Dickinson) and GITR PE (R&D Systems, Abingdon, U.K.) and analyzed by FACS by using Cellquest Pro software (BD). FOXP3 was stained by using the FOXP3 PE staining kit (eBiosciences, San Diego, CA). CD4+CD25− cells were used as a negative control.

Results

Tumor-Draining Lymph Nodes Contain HPV-Specific CD4+ T Cells That Display Regulatory Function. Tumor-draining lymph node biopsies were obtained from three patients with HPV16-positive (C148 and C271) or HPV18-positive (C151) cervical cancers. Lymph node mononuclear cells (LNMC) were isolated and stimulated once with HPV16 or HPV18 E6 and E7 peptides, rested for 2 weeks, and tested for their specificity. Antigen-specific proliferative responses against E6 and/or E7 were detected in all three cultures (Figure 1 A). The LNMC proliferated in the presence of antigen-presenting cells (APCs) pulsed with either peptides or the corresponding

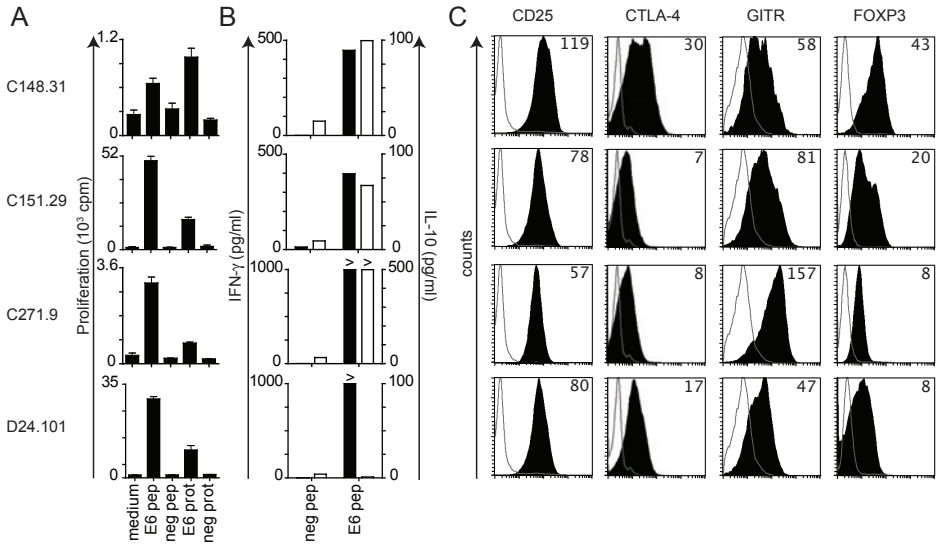

Figure 2. Function and phenotype of patient-derived HPV-specific CD4+ T-cell clones. A) and B) The antigen-specific reactivity and cytokine production of T-cell clones isolated from LNMCs was measured as described in the Fig. 1 legend. The reactivity of HPV16 E6-specific Th1-clone D24.101, which was isolated from a healthy subject, is shown as a control. pep, peptide; prot, protein. C) Expression of the indicated Treg-associated markers was analyzed by flow cytometry 2 weeks after the last specific stimulation (black areas). The numbers in the histograms represent the mean fluorescent intensity (MFI) for each of these stainings. Expression levels of the markers in CD4+CD25− PBMCs are shown as controls (gray lines). The MFI for the CD4+CD25− PBMCs were 6, 3, 9, and 3 for CD25, CTLA-4, GITR, and FOXP3, respectively.

full-length proteins, indicating that the T-cell cultures were capable of recognizing physiological quantities of naturally processed HPV antigens. Antigen-specific proliferation was accompanied by the production of both IFN-γ and IL-10 (Figure 1 B). We focused on these two cytokines, because they represent the outer ends in the spectrum of pro- and anti-inflammatory cytokines.

Limiting dilution of the LNMC cultures resulted in the isolation of HPV-specific T-cell clones that displayed a CD3+, CD4+, CD8 phenotype as determined by flow cytometry (data not shown). These T-cell clones specifically recognized the E6 antigen of HPV16 (patients C148 and C271) or HPV18 (patient C151) and responded against APCs pulsed with either peptide or protein antigen (Figure 2 A). Furthermore, these T cells produced both IFN-γ and IL-10 upon antigen encounter (Figure 2 B) in a manner similar to the polyclonal cultures from which they were derived (Figure 1 B). In contrast, the HPV16 E6-specific CD4+ T-cell clone D24.101, which is representative of HPV-specific CD4 Th1 cells that can be routinely isolated from PBMC cultures of healthy individuals (4, 6, 17, 18), produced IFN-γ but no IL-10. The simultaneous production of IFN-γ and IL-10 by the patient-derived T cells is reminiscent of that by CD4+ regulatory T cells found against EBV (22) in airway hyperreactivity (23) and melanoma (13). Furthermore, we and others observed increased numbers of T cells with regulatory phenotype in the tumor-draining lymph nodes (24) and tumors (25) from cervical cancer patients. We therefore examined our T-cell clones by flow cytometry for markers typically expressed by regulatory T cells: CD25, cytotoxic T cell-associated antigen 4 (CTLA-4), glucocorticoid-induced TNF receptor-family-related gene (GITR), and Foxp3. When compared with the control T-cell clone D24.101, clone C148.31 prominently expressed CTLA-4 and Foxp3, whereas clones C151.29 and C271.9 primarily expressed GITR (Figure 2 C). Overall, the patient-derived T cell clones expressed one or more of these markers but were not characterized by a particular marker profile.

The suppressive capacity of the CD4+ T-cell clones was examined in a coculture experiment with freshly purified CD4+CD25− responder T cells, a standardized APC mixture of five allogeneic EBV-transformed B cell lines, and agonistic CD3-specific antibody (20). Irrespective of differences in their marker profiles, all three patient-derived T-cell clones were able to consistently suppress the proliferation and IFN-γ production of the responder T cells in a cell ratio-dependent manner (Figure 3 A and B and Figure 7). In contrast, the HPV16 E6-specific control T-cell clone D24.101 did not suppress proliferation or IFN-γ production by the responder T cells. Our data demonstrate that cervical cancer-draining lymph nodes harbor HPV-specific CD4+ regulatory T cells.

Suppressive Action of HPV-Specific Regulatory T Cells Depends On Presence of Cognate Antigen. For more in-depth functional analysis of the patient-derived CD4+ T cells, we used assays in which the CD4+CD25− responder T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), and proliferation of these

cells was visualized by flow cytometry. The responder cells were stimulated with the standardized APC mixture and IL-2 in the presence of the patient-derived CD4+ T-cell clones. Notably, the standard APC mixture used in all our suppression assays was composed in such a manner that it expressed all relevant human leucocyte antigen (HLA) class II molecules (see Materials and Methods) and thus could serve as APCs for the different CD4+ T cell clones tested. As indicated by the appearance of peaks with lower CFSE intensity (Figure 3 C Left), the responder T cells strongly proliferated upon stimulation with the allogeneic APCs in the presence of the HPV-specific CD4+ T-cell clones, provided that no HPV peptide antigen was added to the cultures. This proliferative response was indistinguishable from that observed in the absence of regulatory T cells (data not shown). Importantly, in the presence of their cognate antigen, the patient-derived CD4+ T-cell clones did markedly suppress responder cell proliferation (Figure 3 C Right). The possibility of inhibition through crowding was excluded by mitomycin C treatment and irradiation of the patient-derived T cells before their addition to the responder T-cell cultures. Furthermore, control CD4+ T-cell clone D24.101 did not suppress responder-cell proliferation, independent of whether its cognate antigen was added to the culture. Finally, the suppressive action of the patient-derived T-cell clones was unlikely to involve deprivation of responder T cells from IL-2, for instance through consumption by the regulatory T cells, because all assays were performed in the presence of exogenously added IL-2 (300 units/ml).

Even though deprivation of responder cells from IL-2 did not constitute a crucial aspect of the mode of action of our patient-derived CD4+ regulatory T cells in vitro, production of IL-2 by activated T cells can have a decisive impact on the magnitude of the T-cell effector response (26). We therefore tested whether the patient-derived CD4+ T cells could inhibit the production of IL-2 by responder T cells. In this case, the responder T cells used were CD4+ Th1 cells with known specificity and HLA restriction, allowing an experimental set up in which regulator and responder T cells could be stimulated selectively with their cognate antigens. Addition of the target antigen of the responder cells to cocultures of responder and regulatory T cells resulted in secretion of IL-2. No IL-2 production was detected when only the HPV-specific regulatory T cells were stimulated (Figure 4 A), indicating that the IL-2 measured in the culture supernatant was produced exclusively by the responder cells. Importantly, this IL-2 production was inhibited in a dose-dependent manner when the HPV antigen recognized by the regulatory T cells was added. The impact of HPV-specific regulatory T cells on IL-2 production by responder T cells was further visualized in a flow cytometric assay in which IL-2 was detected by intracellular staining and in which responder and regulatory T cells could be discriminated on the basis of CFSE labeling (Figure 4 B). This method allowed us to study whether the lack of IL-2 in the culture supernatants (Figure 4 A) resulted from IL-2 consumption by the regulatory T cells or from blockade of IL-2 production by the responder cells. As shown in Figure 4 B, IL-2 synthesis was confined to the CD4+ Th1 cells and only inhibited when the HPV-specific regulatory T cells were activated.

The inhibitory action of the regulatory T cells was not detected in transwell assays in which responder and regulatory T cells were physically separated (Figure

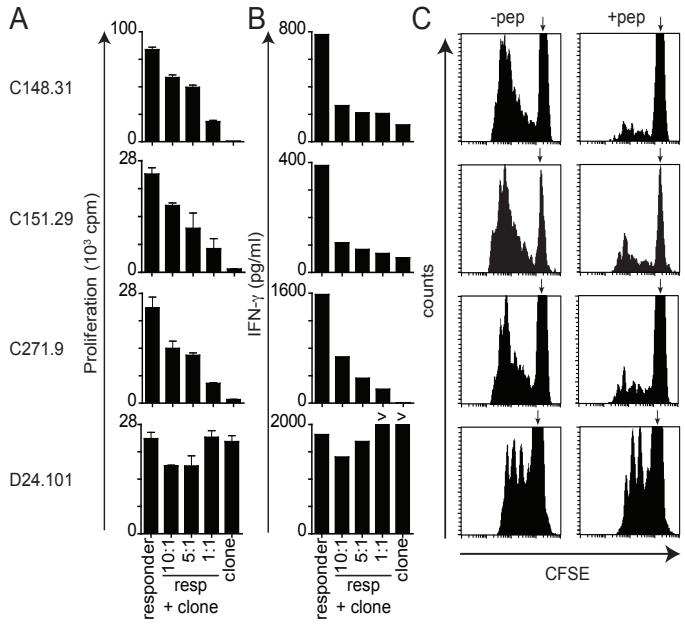

Figure 3. Patient-derived HPV-specific CD4+ T-cell clones display antigen-dependent regulatory function. A) and B) CD4+CD25− responder (resp) T cells (1×10^5) were cultured with the indicated HPV-specific CD4+ T-cell clones at different ratios in 200 µl of medium containing a standardized APC mixture (1×10^4 B-LCL) and 1 µg/ml CD3-specific antibody. After 48h of culture, the allo-response by the responder T cells was analyzed on the basis of [^{3}H]thymidine incorporation and IFN-γ secretion. C) The antigen dependency of regulatory T-cell action was studied by incubating 10^5 CFSE-labeled CD4+CD25− responder T cells with the indicated HPV-specific CD4+ T-cell clones (1:1 ratio) in 200 µl of medium containing the APC mixture and IL-2 (300 units/ml). After 4 days of culture, the allo-specific proliferation of responder T cells was analyzed by flow cytometric analysis of the CFSE intensity. Depicted is responder cell proliferation in the absence or presence, respectively, of 5 µg/ml of clone-specific HPV E6-peptide (pep). CFSE intensity of undivided responder T cells is indicated with arrows above each panel.

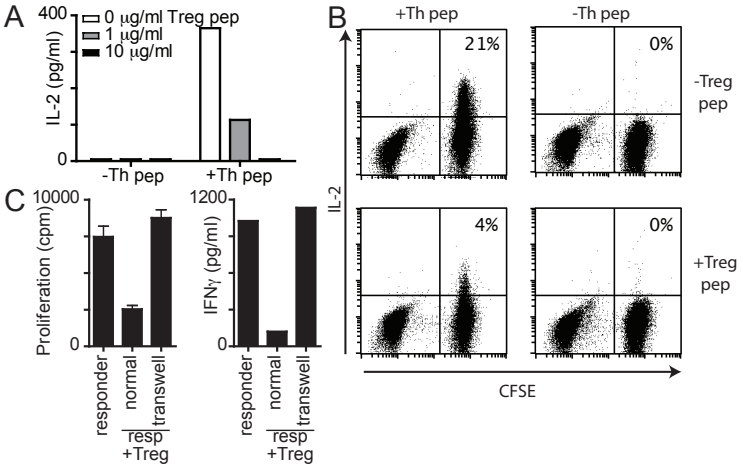

Figure 4. HPV E6–specific regulatory CD4+ T cells suppress IL-2 production by responder T cells. A) HPV–specific CD4+ T cells (1×10^5; clone C148.31) were cocultured with the influenza M1–specific CD4+ Th1 clone #3 at a 1:1 ratio in the presence of an APC mixture (see Fig. 3 legend) capable of presenting the two relevant peptide epitopes. Where indicated, 10 µg/ml of FluM1 peptide (Th pep) and/or 1 or 10 µg/ml of HPV16 E6 peptide (Treg pep) was added to the cultures. Supernatants were collected after 24 h and analyzed for IL-2 content by ELISA. B) HPV–specific CD4+ T cells (clone C271.9) and CFSE–labeled, HIV reverse transcriptase–specific Th1 clone #36 were cocultured at a 1:1 ratio in the presence of APCs capable of presenting the relevant peptide epitopes. Where indicated, 1 µg/ml of HIV reverse transcriptase peptide (Th pep) and/or 10 µg/ml HPV16 E6 peptide (Treg pep) was added to the cultures. Brefeldin A was added to cultures after 1 h, and 26 h later, IL-2 production by the T cells was measured by intracellular cytokine staining and flow cytometry. C) CD4+CD25− responder T cells (2×10^5) were cultured in the outer wells of 24–well transwell plates in medium containing 1 µg/ml anti–CD3 antibody and 2×10^4 APCs, either alone (responder) or with equal numbers of regulatory T cells (resp + Treg). The regulatory T cells were also added to the outer wells, as in a classical suppressor assay (normal), or were separately cultured in the inner wells (transwell). In the latter case, medium in the inner wells was also supplemented with 1 µg/ml anti–CD3 antibody and 2×10^4 APCs. After 56 h of culture, culture supernatant was harvested for analysis of IFN–γ production by ELISA, whereas the cells from the outer and inner wells were separately transferred to 96–well plates and analyzed for their proliferation by [^{3}H]thymidine incorporation.

4 C) nor when supernatant from activated HPV-specific regulatory T-cell cultures was added to responder T cells (data not shown). These data suggest that the action of regulatory T cells on responder T cells depended on close contact between both T-cell subsets and could not be mediated solely by cytokines and/or other soluble factors, in line with other reports (13, 14). Notably, these close-range interactions did not require simultaneous docking of responder and regulatory T cells at the same APC, because the two different EBV-transformed B-lymphoblastoid cell lines (B-LCLs) used as APCs in our assays (Figure 4) expressed the restricting HLA molecule for either the responder T cell or the regulatory T cell.

In summary, the HPV-specific CD4+ regulatory T cells isolated from cervical cancer patients are capable of suppressing proliferation and cytokine (IFN-γ, IL-2) production by responder CD4+ T cells. Their capacity to exert this suppressive effect depends on their activation by their cognate HPV E6 antigen and on close-range interactions with responder T cells.

Isolation of HPV-Specific Regulatory T Cells from Tumor Biopsies. The isolation of HPV-specific regulatory T cells from tumor-draining lymph nodes of patients with cervical cancer suggested that such T cells may also be found in the tumor tissue. Therefore, we performed functional analysis of HPV-specific tumor-infiltrating lymphocyte (TIL) cultures isolated from the tumor biopsies of four patients with HPV16+ cervical cancer (C176, C226, C265, and C331). The six HPV-specific T-cell lines established from these TIL cultures proliferated in the presence of autologous APCs that were pulsed with either their cognate HPV16 E6 peptide or recombinant E6 protein (Figure 5 A). Antigen-specific proliferation was accompanied by secretion of IFN-γ and/or IL-10 (Figure 7A). Of these HPV-specific CD4+ TIL cell lines, four were found to suppress the proliferation of CD4+CD25− responder T cells in a cell ratio-dependent fashion (Figure 5 B; C176.1, C226.1, C265, and C331). In contrast, the other two HPV-specific CD4+ TIL cell lines did not display any suppressor activity in this assay (Figure 5 B; C176.2 and C226.2), despite the fact that they were isolated from the same biopsies as suppressive T-cell lines (C176.1 and C226.1) and secreted both IFN-γ and IL-10 (Figure 8A). Our results therefore indicate that suppressive and nonsuppressive HPV-specific CD4+ T cells coexist in tumors.

We recently performed a study in which healthy subjects were injected intradermally with pools of HPV16 E6 and E7 peptides, after which biopsies from skin areas displaying delayed-type hypersensitivity reactions were taken for examination of the infiltrating T cells (see Materials and Methods). This study gave us the unique opportunity to functionally compare the HPV-specific CD4+ T cells retrieved from cervical cancer biopsies with those isolated from positive skin test biopsies

of healthy subjects. The skin biopsy-derived T cells (P02 and P08) were isolated, propagated, and tested in the same manner as the patient-derived TIL cultures. Similar to the TIL cultures, these CD4+ T-cell cultures proliferated in the presence of autologous APCs pulsed with either their cognate HPV16 E6 peptide or recombinant E6 protein (Figure 6 A) and produced both IFN-γ and IL-10 (Figure 8B). Importantly, however, they were not able to suppress the proliferation of CD4+CD25− responder T cells (Figure 6 B). These data show that the HPV16 E6-specific CD4+ T cells retrieved from delayed-type hypersensitivity skin reactions are functionally distinct from their counterparts isolated from HPV16+ cervical carcinomas.

In conclusion, our data demonstrate that HPV-specific CD4+ regulatory T cells are present in both lymph nodes and tumor tissue of cervical cancer patients, indicating that they are likely to interfere with the anti-tumor immune response at both the induction and effector levels.

Discussion

In this study, we provide a previously unrecognized example of the association between virus-induced cancers and viral antigen-specific CD4+ regulatory T cells. Our search for such CD4+ regulatory T cells, specific for the oncoproteins of HPV16 and HPV18, was prompted by our earlier observations that HPV-positive cervical neoplasia was associated with failure to mount an effective T-cell response against these antigens (5-9). The presence of HPV-specific regulatory T cells at the interface of tumor and immune system, both in the tumor-draining lymph nodes and in the tumor tissue, can explain the development of immunogenic tumors in patients who do not display overt immunodeficiencies.

Although expression of several markers, such as the surface molecules CD25, CTLA-4, and GITR and the transcription factor Foxp3, is reportedly associated with CD4+ T-regulatory function, we found our patient-derived CD4+ T cells to not display one particular marker profile. A CTLA-4highFoxp3highGITRdim T-cell clone and a CTLA-4dimFoxp3dimGITRhigh T-cell clone displayed similar suppressor function (Figs. 2 and 3), indicating that the marker phenotype is, at least in our studies, not a key determinant of CD4+ regulatory T cells. Also, secretion of IL-10 was not uniquely associated with regulatory T cell function, in that tumor-derived T cells lacking prominent IL-10 secretion displayed clear-cut suppressor function (C265 and C331), whereas HPV-specific T cells isolated from healthy skin biopsies lacked suppressor function despite their IL-10 secretion (Figure 6 and Figure 8). Furthermore, we found IL-10 blocking antibodies to not affect the suppressive action of our patient-derived T-cell clones (unpublished data). In accordance to what has been proposed by others, we tentatively conclude that regulatory T cells must be defined primarily through their immunosuppressive function, not on the basis of their phenotype (27, 28). Importantly, the definition of regulatory T cells through function and not by phenotype will make it difficult to directly determine the frequency of all antigen-specific regulatory T cells in peripheral blood, lymphoid organs, or tumors. The use of HLA class II tetramers may aid the detection of the subset of antigen-specific regulatory T cells that display strong and stable expression of Foxp3.

The inhibitory action of the patient-derived CD4+ regulatory T cells depends on the presence of their cognate HPV antigen (Figs. 3 and 4) or alternatively agonistic anti-CD3 antibody. Once activated, these

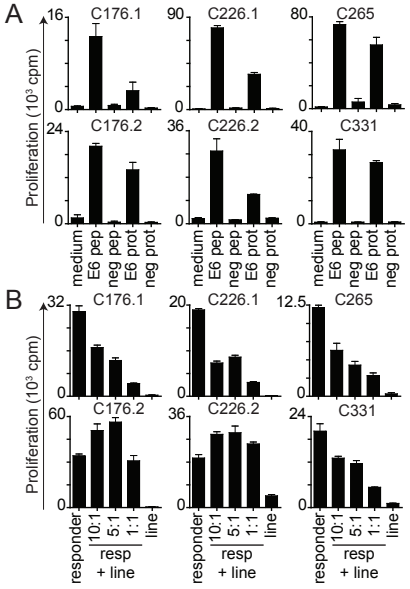

Figure 5. TIL cultures from cancer patients contain HPV-specific regulatory CD4+ T cells. A) The proliferative reactivity of HPV-specific CD4+ T cell lines derived from cervical cancer-derived TIL cultures (C176, C226, C265, and C331) upon stimulation with either HPV E6 peptide or E6 protein was analyzed as described in the Fig. 1 legend. B) The indicated HPV-specific T cell lines were tested for their capacity to suppress proliferation of CD4+ responder T cells in a CD3 antibody-dependent suppression assay as described in the Figure 3 legend.

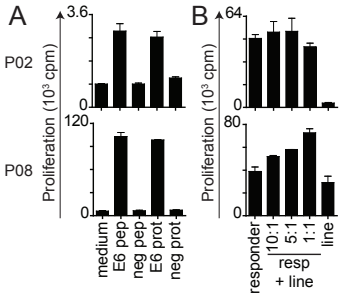

Figure 6. HPV16 E6 skin test biopsy cultures do not contain HPV-specific regulatory T cells. HPV16 E6-specific T cells cultured from a biopsy of the skin test site of two healthy control individuals (P02 and P08) were analyzed for proliferative capacity (A) and capacity to suppress proliferation of CD4+ responder cells (B) according to procedures described in the Figure 5 legend.

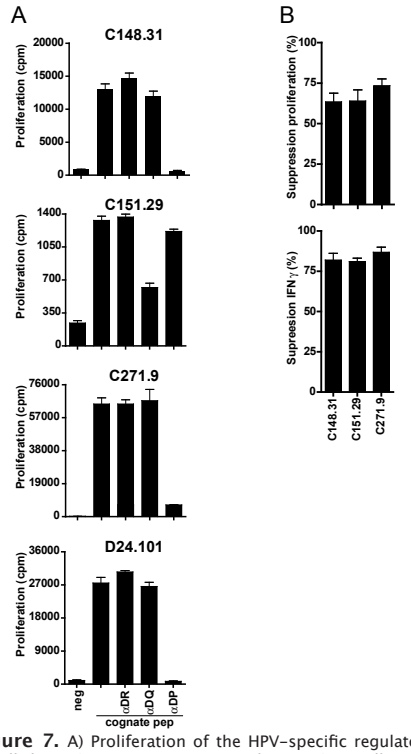

Figure 7. A) Proliferation of the HPV-specific regulatory T-cell clones (C148.31, C151.29, and C271.9) as well as the HPV-specific helper T-cell clone (D24.101) when stimulated with their cognate peptide antigen in the absence and presence of MHC class II blocking antibodies. B) Consistency and variation in the capacity of the regulatory T-cell clones to suppress proliferation and IFN-γ production of naïve CD4+CD25- responder T cells. Depicted are the mean and standard deviation of four different experiments.

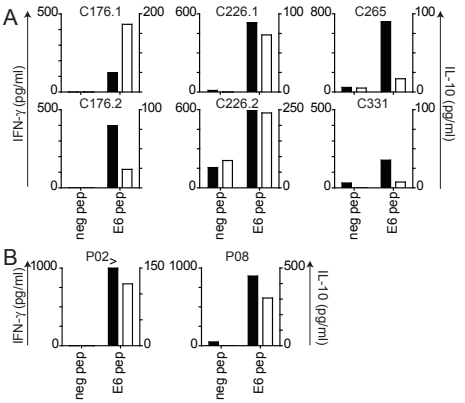

Figure 8. HPV-specific IFN-γ (filled bars) and IL-10 (open bars) production by the TIL lines depicted in Figure 5 (A) and the skin biopsy lines depicted in Figure 6 (B) were analyzed as described in the Figure 1 legend.

regulatory T cells can exert their inhibitory function against responder T cells with various antigen specificity, including allo-reactive T cells (Figure 3) and T cells specific for antigens from HIV-1 or influenza virus (Figure 4). In this respect, their functional features are comparable to those of previously described tumor antigen-specific regulatory T cells isolated from mice (12, 29) or cancer patients (13, 14, 21). However, the HPV-specific CD4+ regulatory T cells differ essentially from their previously described counterparts in that they are specific for tumor-specific antigens of viral origin. Our data therefore indicate that suppression of anti-tumor immunity by CD4+ regulatory T cells is not limited to tumor-associated self antigens but extends toward foreign antigens encoded by tumor viruses. Notably, the viral antigens concerned are the prime components of all therapeutic vaccines against cervical cancer that are currently under development (30-32). Although such vaccines are designed to enhance CD4+ and CD8+ T cell effector immunity against the E6 and E7 oncoproteins of HPV16 and/or 18, the presence of preexisting E6 and E7-specific CD4+ regulatory T cells in lymph nodes and tumors from cervical cancer patients brings forward the possibility that vaccination might also, or instead, result in activation and expansion of this regulatory T-cell subset. This risk was recently illustrated in an elegant mouse study, which showed that vaccination of tumor-bearing hosts harboring a mixture of antigen-specific responder and regulatory T cells resulted in concomitant expansion of both T-cell populations. Importantly, the net effect of vaccination in this setting was failure of the anti-tumor immune response (29). Our data argue that this scenario must be taken into account when vaccinating cervical cancer patients with E6/E7-specific vaccines and indicate that strategies to eliminate or disarm regulatory T cells before vaccination, which are now widely considered in the context of modalities that aim at inducing effective immune responses against tumor-associated autoantigens (33-36), should also be considered for immunotherapeutic strategies against cancers with viral etiology.

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