View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Catalogo dei prodotti della ricerca

ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Gynecologic Oncology

Gynecologic Oncology 89 (2003) 271–280

www.elsevier.com/locate/ygyno

Induction of tumor-specific cytotoxicity in tumor infiltrating lymphocytes by HPV16 and HPV18 E7-pulsed autologous dendritic cells in patients with cancer of the uterine cervix

Alessandro D. Santin, M.D.,[a,*] Stefania Bellone, Ph.D.,[a] Michela Palmieri, M.S.,[a] Barbara Bossini, M.S.,[a] Juan J. Roman, M.D.,[a] Martin J. Cannon, Ph.D.,[b] Eliana Bignotti, Ph.D.,[c] Stefania Canè, Ph.D.,[c] and Sergio Pecorelli, M.D.[c]

[a] *Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, University of Arkansas for Medical Sciences, Little Rock, AR 72205-7199, USA*

[b] *Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, AR 72205-7119, USA*

[c] *Division of Gynecologic Oncology, University of Brescia, Brescia, Italy*

Received 31 July 2002

Abstract

Objective. To evaluate the potential of autologous dendritic cells (DC) pulsed with HPV16 and HPV18 E7 oncoprotein in restoring tumor-specific cytotoxicity in populations of tumor infiltrating lymphocytes (TIL) for adoptive immunotherapy of cervical cancer patients.

Methods. Full-length E7-pulsed DC-stimulated $CD8^+$ T cells derived from peripheral blood (PBL) and from tumor tissues (TIL) were tested and compared for their ability to induce a HLA class-I-restricted cytotoxic T lymphocyte (CTL) response against autologous tumor cells. In addition, in order to correlate cytotoxic activity by CTL with a particular lymphoid subset, analysis of surface antigens and intracellular CD3 ζ chain and two-color flow cytometric analysis of intracellular cytokine expression (IFN-γ vs IL-4) at the single cell level were performed.

Results. DC stimulation induced powerful cytotoxicity against autologous tumor target cells by TIL-derived $CD8^+$ T cells from all three cervical cancer patients, while autologous Epstein–Barr virus-transformed lymphoblastoid cell lines were not lysed. Killing of autologous tumor cells was higher by $CD8^+$ T cells from TIL compared to PBL ($P > 0.01$) and was more strongly inhibited by anti-HLA class I MAb ($P > 0.05$). Phenotypically, all CTL populations were $CD3^+/CD8^+$, with higher levels of CD56 expression by TIL-derived CTL. Finally, although a marked Type 1 cytokine bias (i.e., IFN-γ^{high}/IL-4^{low}) was observable in both PBL- and TIL-derived DC-stimulated $CD8^+$ T cell populations, TIL-derived $CD8^+$ T cells showed a higher percentage of IFN-γ-positive cells compared to PBL.

Conclusions. Full-length E7-pulsed DC can consistently restore strong $CD8^+$ CTL responses against autologous HPV16- and HPV18-infected cervical cancer cells. DC-stimulated TIL may represent a superior source of tumor-specific CTL compared to PBL for adoptive T cell immunotherapy of patients harboring metastatic or recurrent cervical cancer refractory to standard treatment modalities.

© 2003 Elsevier Science (USA). All rights reserved.

Keywords: Cervical cancer; Tumor infiltrating lymphocytes; IFN-γ; IL-4

Introduction

Management of disseminated carcinoma of the cervix no longer amenable to control with surgery or radiation therapy has not improved significantly with the progress of modern chemotherapy, with a 1-year survival rate of 10% [1]. Novel therapeutic strategies effective in the treatment of metastatic and/or recurrent disease are still desperately needed. Human papillomavirus (HPV)[1] infection from high-risk genotypes

* Corresponding author. UAMS Medical Center, Division of Gynecologic Oncology University of Arkansas for Medical Sciences, 4301 W. Markham, Little Rock, AR 72205-7199. Fax: +1-501-686-8091.

E-mail address: santinalessandrod@uams.edu (A.D. Santin).

[1] Abbreviations used: HPV, human papillomavirus; DC, dendritic cells; PBL, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cells; CTL, cytotoxic T lymphocytes; TIL, tumor infiltrating lymphocytes;

0090-8258/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0090-8258(03)00083-0

(i.e., HPV16 and HPV18) represents the most important risk factor for developing cervical cancer and more than 90% of cervical cancers contain integrated viral genes encoding two specific oncoproteins (E6 and E7)[2,3]. Although the interactions between the host immune system and HPV-infected cells are still not completely understood, cell-mediated immunity seems to play an important role in the control of HPV-associated malignancy. In support of this view, substantial evidence has shown increased incidence of associated genital cancer and a more rapid progression of HPV-infected tumors in immunosuppressed patients, while only a minority of genital HPV infections result in the development of cancer in otherwise healthy individuals [4–6]. Infiltrating $CD4^+$ (helper) and $CD8^+$ (cytotoxic/suppressor) T cells and NK cells have been observed in spontaneously regressing HPV-associated warts [7,8], and the degree of lymphoid cell infiltration of the neoplastic cervix [9] and the ability of such reactive cells to secrete the immunostimulatory and antiviral cytokine IFN-γ [10] have been reported to be independent factors for disease-free survival in cervical cancer patients. Better prognosis in early-stage HPV-positive cervical carcinoma than HPV-negative disease has been previously reported [11]. Studies in animals have demonstrated that immunized animals are protected from papillomavirus infections and from transplanted tumor cells expressing HPV E6/E7 viral proteins [12–14]. Finally, the detection of in vivo-primed HPV-specific T cells as well as the in vitro generation of HPV-specific cytotoxic T lymphocytes (CTL) has recently been reported in cervical cancer patients [15–18].

Dendritic cells (DC) are the most potent professional antigen presenting cells (pAPC) for activation of T and B cell immune responses [19] and recently the combination of GM-CSF and IL-4 has been shown to generate large numbers of DC for the manipulation of human T cell responses [20,21]. In the last few years, human phase I trials have been initiated using peptide/protein pulsed DC, and promising results have been reported for several human tumors, without significant side effects from the vaccinations [22–26].

One of the most important components of successful tumor-specific adoptive immunotherapy is the activation and expansion of large numbers of tumor-specific T lymphocytes. In support of this view, tumor infiltrating lymphocytes (TIL) have been shown to be a reliable source of tumor-specific CTL precursors for effective adoptive immunotherapy [27,28]. However, in several studies, lymphocytes derived from TIL have been shown to be defective in several immunologic functions, including (1) the ability to proliferate in the presence of mitogens, phorbol esters, or low doses of IL-2, (2) cytotoxic activity against autologous or allogeneic tumor cells, (3) effector activity in assays of natural killing, and (4) expression of cytoplasmic CD3 ζ chain, which is essential for T cell receptor (TcR) signaling [reviewed in 29,30]. Although the mechanisms responsible for these impaired immune responses are yet not completely understood, in some cases, poor immune responses by recently explanted T lymphocytes could be normalized upon in vitro culture with recombinant IL-2 [31] or by T cell stimulation with anti-CD3 and anti-CD28 antibodies [32]. It is therefore possible that when properly reactivated by mature tumor antigen-pulsed DC, which are endowed with high expression of HLA class I and II antigens and costimulatory molecules [33], lymphocytes derived from TIL may be a superior source of tumor-specific CTL for adoptive T cell immunotherapy for cervical cancer.

In this study we have evaluated and compared the phenotype and function of HPV16 and HPV18 E7-pulsed autologous DC-stimulated $CD8^+$ T lymphocytes derived from PBL and TIL of cervical cancer patients. Here we show for the first time that full-length E7-pulsed fully mature DC have the potential to consistently reverse the anergic state of TIL populations derived from cervical cancer patients and that TIL constitute a superior source of tumor-specific $CD8^+$ CTL compared to PBL for potential use in adoptive immunotherapy of cervical cancer patients unresponsive to standard salvage treatment modalities.

Materials and methods

Patients

Three patients who had undergone radical hysterectomy for invasive cervical cancer provided tumor tissue and peripheral blood lymphocytes. Specimens were obtained at the time of surgery through the Division of Gynecologic Oncology and the Pathology Department at the University of Arkansas for Medical Sciences (UAMS), Little Rock, Arkansas, under approval of the Institutional Review Board. HPV typing was performed on fresh tissue biopsy and on the derived fresh cultures by PCR using sequence-specific primers for HPV -16, -18, -31, -33, -52b, and -58 [34]. Patients 1 and 2 had stage IB2 squamous cell carcinomas positive for HPV 16 and were ages 35 and 37, while patient 3 had stage IB2 adenocarcinoma positive for HPV 18 and was age 31.

Tumor cell lines

Fresh tumor cells were obtained from multiple punch biopsies from all patients. Biopsies were divided into two parts, for histopathologic evaluation and for in vitro studies. Fresh tumor cell lines were maintained in serum-free keratinocyte medium (KSFM, Invitrogen, Grand Island, NY), supplemented with 5 ng/ml of epidermal growth factor and 35 to 50 μg/ml of bovine pituitary extract at 37°C, 5% CO_2. Briefly, single cell suspensions were obtained by processing solid tumor samples under sterile conditions at room tem-

HLA, human leukocyte antigens; IFN-γ, interferon-gamma; IL-4, interleukin-4.

perature. Viable tumor tissue was mechanically minced in RPMI 1640 to portions no larger than 1–3 mm^3 and washed twice with RPMI 1640. The portions of minced tumor were then placed into 250-ml trypsinizing flasks containing 30 ml of enzyme solution [0.14% Collagenase Type I (Sigma, St. Louis, MO) and 0.01% DNase (Sigma, 2000 KU/mg)] in RPMI 1640 and incubated on a magnetic stirring apparatus overnight at 4°C. Enzymatically dissociated tumor was then filtered through 150-μm nylon mesh to generate a single cell suspension. The resultant cell suspension was then washed twice in RPMI 1640 plus 10% autologous plasma. All experiments were performed with fresh or cryopreserved tumor cultures which had at least 90% viability and contained >99% tumor cells.

Collection and isolation of peripheral blood lymphocytes (PBL) from cervical cancer patients

Peripheral blood was collected in heparinized tubes and processed as previously described [34], to separate the mononuclear leukocytes from red blood cells by Ficoll–Hypaque density gradient centrifugation. The leukocytes were subsequently harvested, washed twice in phosphate-buffered saline (PBS, pH 7.2), and either seeded in six-well plates in AIM-V medium (Invitrogen) for DC generation as described below or resuspended in RPMI 1640 plus 5% human AB serum (Gemini BioProducts, Calabasas, CA) before staining with monoclonal antibodies (MAb).

Isolation of TIL

Specimens were obtained at the time of diagnosis or surgery. Briefly, tumor cells and TIL single cell suspensions were obtained by processing solid tumor samples under sterile conditions as described above. Cell suspensions containing tumor cells and TIL were then washed twice in RPMI 1640 plus 10% autologous plasma, placed on discontinuous Ficoll–Hypaque (75/100%) density gradients, and centrifuged again to separate and harvest TIL and tumor cells as previously described [35]. Enriched TIL preparations were then washed twice in RPMI 1640 plus 10% autologous plasma and either immediately activated with PMA and ionomycin for intracellular cytokine studies or incubated overnight at 37°C in tissue culture flasks before collecting the TIL from the nonadherent population.

DC cultures and generation of HPV E7-specific T cells

The derivation of DC from the patients' PBMC, and their subsequent use for generation of HPV E7-specific T cells, was carried out essentially as described [34]. E7-specific $CD8^+$ T cells were derived from cervical cancer patients from lymphocytes obtained from PBL and tumor tissue (TIL). Briefly, DC were generated from plastic-adherent PBMC by culture in AIM-V medium plus 800 U/ml of GM-CSF (Immunex, Seattle, WA) and 500 U/ml of IL-4 (R & D Systems, Minneapolis, MN). Cultures were fed by half-changes of AIM-V plus GM-CSF and IL-4 every 2 days. Final maturation of monocyte-derived DC was induced by exposure during the last 48 h of culture (i.e., days 6 to 8) to TNF-α (1000 U/ml) IL-1β (500 U/ml) (R & D Systems), and PGE2a (0.5 μM/ml) (Sigma). The DC purity (i.e., cells strongly expressing $HLA\text{-}DR^+$, $CD86^+$, $CD83^+$, $CD80^+$, $CD40^+$, and $CD14^-$) ranged from 62 to 90% of the total cell population, as previously characterized by our laboratory [33]. After final maturation, DC were harvested for pulsing with HPV16 or 18 E7 oncoproteins generated using previously characterized plasmids encoding glutathione S–transferase (GST) E7 fusion proteins [34]. The cationic lipid DOTAP (Boehringer Mannheim, Indianapolis, IN) was used to deliver the HPV16 or 18 E7 proteins into cells as described [34]. HPV E7-specific CTL were generated by culturing responder PBMC and TIL ($10–20 \times 10^6$ cells/well in 6-well culture plates) (Costar, Cambridge, MA) in AIM-V with E7-pulsed autologous DC (ratios from 20:1 to 30:1 responder PBMC/TIL: DC). The cultures were supplemented with recombinant human IL-2 (10 U/ml of Aldesleukin, Chiron Therapeutics, Emeryville, CA) and restimulated once with E7-pulsed DC after 10–14 days. At day 21–28, $CD8^+$ T cells were separated from the bulk cultures by positive selection with CD8 Dynabeads (Dynal Inc., Lake Success, NY) and further expanded in number for 5–7 days using autologous or allogeneic irradiated PBL (5000 cGy) (1×10^6 cells/well) and anti-CD3 MAb (0.2 μg/ml) plus 5% autologous plasma and 100 U/ml of IL-2 in 24-well plates (Costar), before being assayed for phenotype characteristic and CTL activity.

Flow cytometric analysis of surface antigens, intracellular CD3 ζ chain, and cytokines by cervical tumor-specific CTL from PBL and TIL

Flow cytometric analysis of superficial antigens and intracellular cytokine expression was conducted essentially as previously described [36]. Briefly, flow cytometry for superficial antigen expression analysis was performed using directly conjugated MAbs against the following markers: CD3, pan T cells; CD4, T helper/inducer; CD8, T cytotoxic/suppressor; CD19, B cells; CD56, NK/K cells; CD25, the IL-2 receptor; anti-HLA-DR; and anti-TcR α/β (all from Becton–Dickinson, San Jose, CA). Control isotype-matched FITC- or PE-conjugated MAb were also obtained from Becton–Dickinson. For evaluation of the level of CD3ζ chain in $CD8^+$ T cells, PBL and TIL before and after in vitro stimulation with HPV16 or HPV18 E7-pulsed DC were harvested, stained with anti-CD8-FITC (Becton–Dickinson), washed, and fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. Cells were then washed and permeabilized by incubation in PBS plus 1% BSA (A-3059, Sigma) and 0.5% saponin (S-7900, Sigma) for 10 min at room temperature. Experimental and control cells were stained with anti-TcRζ-PE (2H2D9-IgG1k) or

isotype control (Coulter, Marseille, France). After staining, cells were washed twice with PBS plus 1% BSA and 0.5% saponin, once with PBS plus 0.5% BSA, and fixed a second time with 2% paraformaldehyde in PBS. All analyses were conducted with a FACScan, utilizing Cell Quest software (Becton–Dickinson).

For intracellular cytokine staining for IFN-γ and IL-4 expression, $CD8^+$ T cells obtained from PBL and TIL at the beginning of culture were activated with 50 ng/ml PMA and 500 ng/ml of ionomycin for 6 h; 10 μg/ml of Brefeldin A was added for the final 3 h of incubation. Controls (nonactivated cultures) were incubated in the presence of Brefeldin A only. Cells were superficially stained with anti-CD8-FITC or anti-CD8-PE MAb and then washed and permeabilized by incubation in PBS plus 1% BSA and 0.5% saponin for 10 min at room temperature. Activated and control cells were stained with FITC-anti-IFN-γ and/or PE-anti-IL-4 and isotype-matched controls (FITC-anti-Igγ2a and PE-anti-Igγ1) (Becton–Dickinson). Purified DC-stimulated $CD8^+$ T cell populations (i.e., day 21–28 of culture) were also analyzed by two-color flow cytometry for simultaneous expression of IFN-γ and IL-4 at the single cell level. After staining, cells were washed twice with PBS plus 1% BSA and 0.5% saponin, once with PBS plus 0.5% BSA, and fixed a second time with 2% paraformaldehyde in PBS.

Cytotoxic activity

A 6-h chromium (^{51}Cr) release assay was performed as previously described [34] to measure the cytotoxic reactivity of DC-E7-stimulated T lymphocytes. The EBV-transformed lymphoblastoid B cell line (LCL) derived from the same cancer patient that provided the HPV naturally infected primary tumor cell targets was established by coculture of PBMC with EBV-containing supernatant from the B95.8 cell line in the presence of 1 μg/ml of cyclosporin A (Sandoz, Camberley, UK) and was maintained in RPMI 1640 supplemented with 10% human AB serum. To determine the molecular basis of target cell lysis, ^{51}Cr-labeled tumor targets were preincubated with MAbs specific for monomorphic HLA class I (W6/32, 50 μg/ml) or isotype control MAb. The effector cells and ^{51}Cr-labeled targets were then incubated in a final volume of 200 μl/microwell at 37°C with 6% CO_2.

Statistical analysis

Data were analyzed using the Student's *t* test. All data were expressed as mean percentages of positive cells ± standard deviation (SD). In all tests, the difference was considered significant when *P* values were less than 0.05.

Table 1
Subpopulations of mononuclear cells in PBL and TIL from cervical cancer patients

Specific MAb	% Phenotype					
	Patient 1		Patient 2		Patient 3	
	PBL	TIL	PBL	TIL	PBL	TIL
CD3	74	42	69	49	66	39
CD4	50	20	46	21	44	18
CD8	24	22	22	25	23	22
CD19	13	3	15	3	12	2
CD56	9	3	17	2	9	3
CD8/CD56	5	2	8	2	6	1
CD4/CD8 RATIO	2.1	0.9	2.1	0.8	1.9	0.8

Values are percentages of PBL and TIL that are positive for specific markers used in the cell staining before stimulation with E7-pulsed autologous dendritic cells.

Results

Phenotypic analysis of lymphocytes from peripheral blood and tumor tissue

Flow cytometric analysis was used to characterize the phenotype of the populations of lymphocytes obtained from PBL and TIL before and after the in vitro stimulation with autologous HPV16 or HPV18 E7-loaded DC. The proportions of $CD3^+$, $CD8^+$, and $CD4^+$ T cells in PBL and TIL from the three cervical cancer patients evaluated in this study before DC activation are described in Table 1. $CD3^+$ T cells were the major lymphocyte population in each tissue, with the percentage of $CD4^+$ T cells greater than the percentage of $CD8^+$ T cells in PBL ($P < 0.01$) (Table 1). In contrast, in all three patients, TIL contained a significantly higher percentage of $CD8^+$ T cells (Table 1). The PBL had a $CD4^+$ to $CD8^+$ ratio of 2:1, while TIL showed a ratio of 0.8:1 (Table 1). Starting lymphocyte populations were also different in the percentage of CD56- and CD19-positive lymphocytes, with PBL containing more $CD56^+$ and $CD19^+$ cells than TIL ($P < 0.05$ and $P < 0.01$ for CD56 and CD19, respectively). The expression of CD56 on T lymphocytes was further analyzed by two-color immunofluorescence. By this technique, the $CD8^+$ T cells were compared for coexpression of CD56. PBL contained more $CD8^+/CD56^+$ T cells than TIL (Table 1, $P < 0.05$).

After 21–28 days of culture of HPV16 or HPV18 E7-pulsed DC-stimulated PBL and TIL, the striking majority of cells were found to be TcR-$\alpha\beta^+/CD3^+$ T cells (93–98%). PBL contained more $CD4^+$ T cells (range, 58–89%) than $CD8^+$ T cells. In contrast, in the TIL populations derived from all three patients, $CD8^+$ T cells (range, 55–78%) were predominant over $CD4^+$ T cells. Enriched populations of $CD8^+$ T cells were isolated at this time and analyzed for expression of CD56 after a further 2 or more weeks of culture. A variable proportion of CD8/CD56 antigen-positive cells was detected in all $CD8^+$ T cells derived from PBL and TIL. However, as representatively shown for pa-

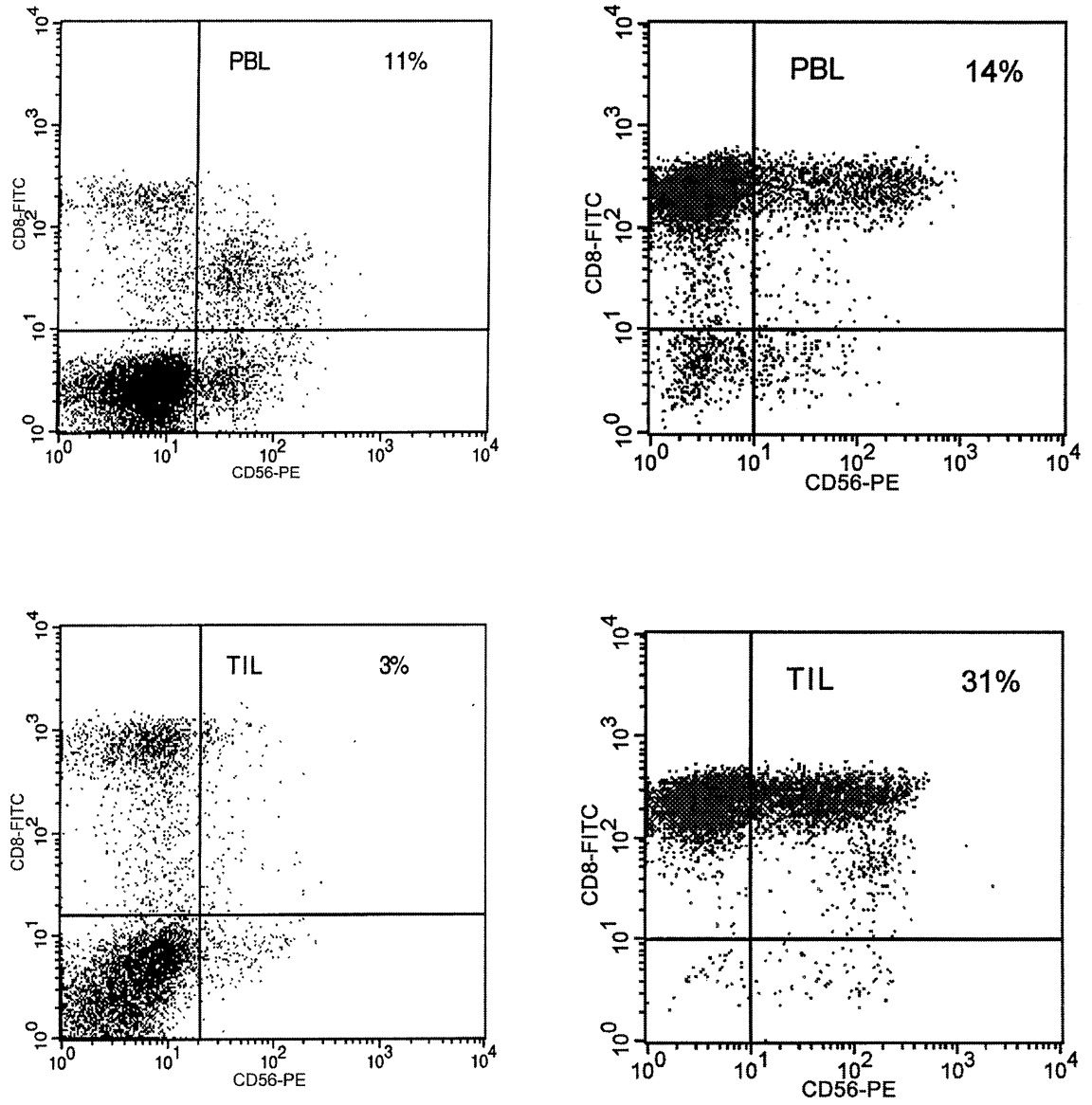

Fig. 1. Two-color flow cytometric analysis of CD56 expression by $CD8^+$ T cells in PBL and TIL from a representative patient before and after in vitro stimulation with full-length HPV16 E7-loaded DC. Numbers in the upper right quadrant represent the percentages of $CD8^+$ $CD56^+$ T cells. (Left) PBL and TIL before DC stimulation. (Right) PBL and TIL after DC stimulation.

tient 1 (Fig. 1), HPV16 or HPV18 E7-DC-stimulated TIL consistently expressed a higher percentage of $CD8^+$/$CD56^+$ T cells than PBL ($P < 0.01$). Further analysis revealed the populations of $CD8^+$ T cells from PBL and TIL to be $CD25^+$, HLA-DR^+, and $CD16^-$ (data not shown).

Expression of intracellular TcR ζ chain in cervical tumor antigen-stimulated T cells

To evaluate whether the expression levels of TcR ζ chain vary significantly in PBL and TIL collected from cervical cancer patients before and/or after DC stimulation, we again took advantage of flow cytometry. As shown in Table 2, the mean fluorescence intensity (MFI) of TcR ζ chain expression was significantly higher in $CD8^+$ T cells derived from PBL than in those derived from TIL ($P < 0.05$) in all three patients. However, after DC stimulation and expansion no significant differences were found in TcR ζ chain expression among $CD8^+$ T cells derived from PBL compared to TIL (data not shown).

Tumor-specific cytotoxic responses in $CD8^+$ T cells derived from PBL and TIL

Cytotoxicity assays on HPV16 or HPV18 E7-loaded DC-stimulated PBL and TIL were conducted after a minimum of 2 weeks following separation of pure populations of

Table 2
Expression of TcR ζ chain in $CD8^+$ T cells derived from PBL and TIL of cervical cancer patients

	Patient 1				Patient 2				Patient 3			
	Before		After		Before		After		Before		After	
	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI
PBL	100	41	80	11	100	49	79	13	100	36	87	12
TIL	98	16	78	9	96	11	69	8	99	14	69	9

Percentage and mean fluorescence intensity (MFI) of TcR ζ chain-positive $CD8^+$ T cells before and after in vitro stimulation with E7-pulsed autologous dendritic cells. The difference in TcR ζ chain expression in PBL versus TIL before DC stimulation was significant at $P > 0.05$. No significance difference in TcR ζ chain expression was noted in PBL versus TIL after DC stimulation.

$CD8^+$ T cells. The results in Fig. 2 represent the mean of not less than three assays for each patient. Cytototoxic activity of DC-stimulated $CD8^+$ T cells ranged from 28 to 46%, mean 38% (PBL), and from 38 to 63%, mean 53% (TIL), for patient 1; from 18 to 36%, mean 28% (PBL), and from 33 to 58%, mean 49% (TIL), for patient 2; and from 26 to 40%, mean 33% (PBL), and from 54 to 73%, mean 63% (TIL), for patient 3. Blocking studies indicated that in all cases tumor-specific lysis by $CD8^+$ T cells was inhibited by MAb specific for HLA class I, the range of inhibition being from 18 to 57% (PBL) and from 38 to 84% (TIL) for the three patients. In all cases low or negligible cytotoxicity was observed against autologous EBV-transformed LCL and against natural killer-sensitive K562 cells (Fig. 2). In contrast with the tumor-specific cytotoxicity consistently observed with DC-stimulated $CD8^+$ T cells from TIL, populations of freshly isolated TIL showed no cytotoxicity against autologous tumor targets (data not shown).

Intracellular cytokine expression by cervical cancer-specific T cells

To determine the pattern of Type 1 and Type 2 cytokine expression in the populations of $CD8^+$ T lymphocytes collected from PBL and TIL, two-color flow cytometric analysis of intracellular IFN-γ or IL-4 expression by $CD8^+$ T cells was performed before and after in vitro culture with HPV16 or HPV18 E7-loaded autologous DC. As described in Table 3, at the beginning of culture, significantly more $CD8^+$ T cells from TIL expressed IFN-γ than PBL-derived $CD8^+$ T cells following PMA and ionomycin activation ($P < 0.05$). In contrast, IL-4 expression was found at low, but significantly higher levels in $CD8^+$ T cells from PBL ($P < 0.05$) when compared to TIL (Table 3). When pure populations of $CD8^+$ T cells were tested after stimulation with E7-loaded DC and further in vitro expansion, significantly higher numbers of $CD8^+$ T cells from TIL were found to contain intracellular IFN-γ compared to $CD8^+$ T cells derived from PBL (Table 3). IL-4 expression was again significantly higher in $CD8^+$ T cells derived from PBL when compared to IL-4 expression by $CD8^+$ T cells obtained from TIL, with higher numbers of double positive (i.e., IFN-γ^+/IL-4^+) $CD8^+$ T cells from PBL (Table 3). Similar results were consistently obtained in several repetitive analyses for all patients. Unactivated (i.e., resting) $CD8^+$ T cells failed to stain for IFN-γ or IL-4, and similarly, FITC-anti-IgG2a and PE-anti-IgG1 isotype controls did not stain either activated or unactivated $CD8^+$ T cells (data not shown).

Discussion

Despite the presence of a lymphocytic infiltrate constituted of Th1 cytokine expressing antigen-experienced $CD8^+$ T cells [9,10,36], many human tumors, including cervical cancers, grow relentlessly, suggesting that these preferentially recruited lymphocyte populations may eventually become functionally suppressed in vivo [29–32]. The mechanisms responsible for this tumor-induced suppression are not completely understood but may include the release of immunosuppressive cytokines, such as TGF-β, IL-10 [reviewed in 29,30], or vascular endothelial growth factor [37] by infiltrating mononuclear cells and tumor cells, or the incompetent or defective antigen presentation by antigen presenting cells that fail to offer adequate help for inducing or maintaining effective immune responses [38].

Dendritic cells are the most potent antigen presenting cells known in humans and play a crucial role during the priming and reactivation of antigen-specific immune responses [19–21]. Because the E6 and E7 transforming oncoproteins of the high-risk genotypes of human papillomavirus (i.e., HPV16 and HPV18) are constantly expressed in the vast majority of cervical cancers, E6 and/or E7 could be ideal candidates as potential tumor-specific targets for cervical cancer immunotherapy [15–18]. However, cervical cancer patients are often severely immunocompromised due to multiple courses of chemotherapy and/or radiation therapy during tumor progression [39–41]. Adoptive infusions of tumor-specific in vitro-activated T cells, which avoid the potential problems associated with inducing a CTL response in vivo, might therefore be a more effective approach for control of tumor growth in patients with cervical cancer refractory to standard treatment modalities. Large numbers of Type 1 cytokine-expressing and antigen-experienced

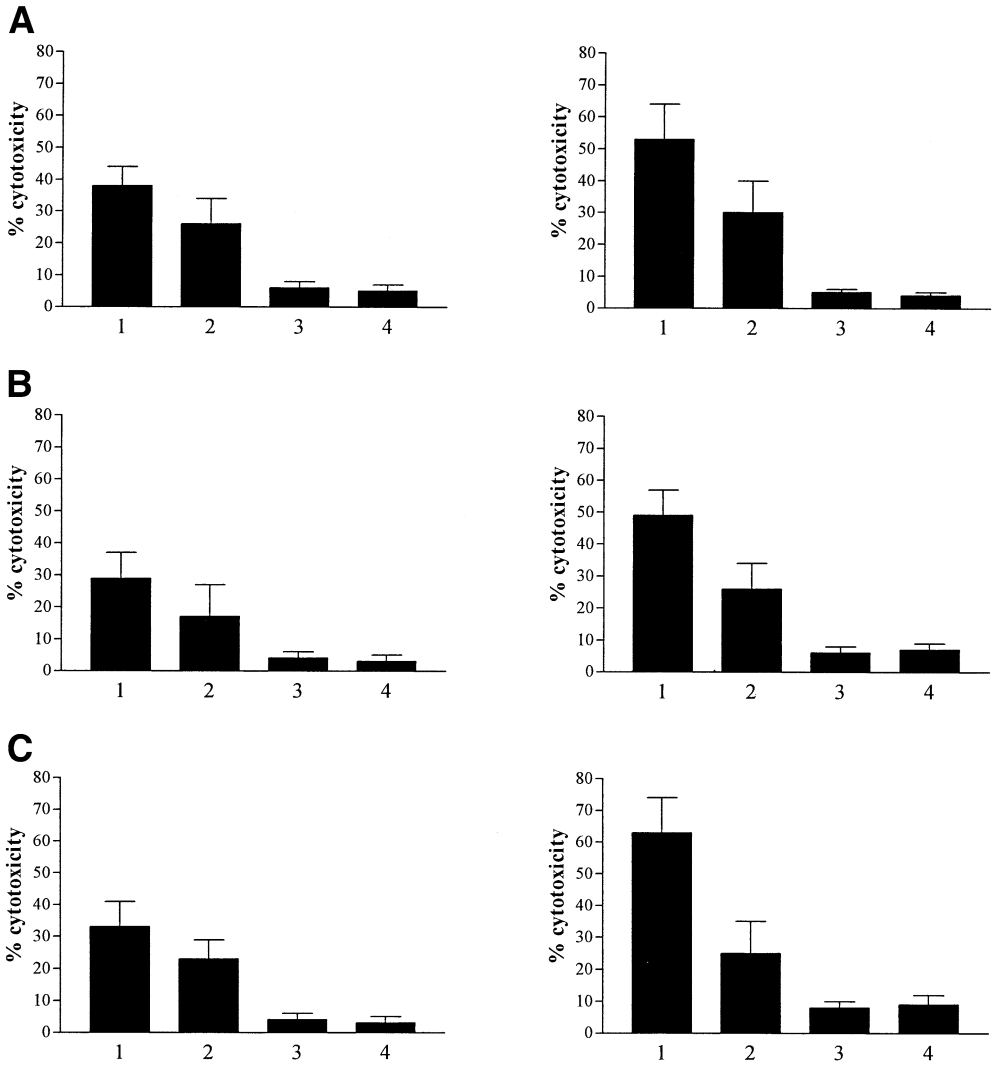

Fig. 2. Tumor-specific CD8$^+$ CTL responses induced in PBL (left) and TIL (right) by full-length HPV16/18 E7-loaded DC in three cervical cancer patients, measured in a 6-h ^{51}Cr-release assay. Percentage of lysis (± standard deviation) at a 20:1 effector:target cell ratio is shown. Anti-HLA class I blocking antibody (W6/32) and isotype control MAb (data not shown) were used at 50 μg/ml. Patients 1 (A), 2 (B), and 3 (C); 1, autologous tumor; 2, autologous tumor+W6/32 anti-class I MAb; 3, autologous LCL; 4, K562.

CD8$^+$ T cells are commonly detected in TIL relative to PBL, suggesting that TIL may be a favorable source of CD8$^+$ T cells for immunotherapy. However, the immunocompetence of human TIL and the possibility of consistently restoring their tumor-specific lytic activity in vitro remains an unresolved issue.

In this study, as a basis for the development of more effective immunotherapy protocols for the treatment of metastatic disease, we performed a careful functional analysis of lymphocytes derived from PBL and TIL before and after in vitro stimulation with tumor antigen-pulsed autologous mature DC.

We found TIL to be a superior source of tumor-specific CTL compared to PBL. Although TIL were not cytotoxic immediately after collection, induction of higher cytotoxic activity against autologous HPV16 or HPV18 tumor cells was consistently noted in E7-loaded DC-stimulated CD8$^+$ T cells from TIL when compared to CD8$^+$ T cells derived from PBL. The lack of significant cytotoxicity against HLA-identical autologous LCL confirmed that, although these TIL-derived CTL were highly cytolytic against autologous tumor cells, they failed to kill autologous control target cells expressing different antigens. Furthermore, the fine specificity of the TIL-derived populations of CTL was also confirmed by the significantly higher block in cytotoxic activity detected using anti-HLA class I MAb when compared to PBL-derived CTL and by their minor cytotoxic activity against K562 natural killer-sensitive target cells. In this regard, the lack of a complete block in cytotoxicity by anti-HLA class I MAb can be at least partially explained by the presence of a heterogenous population of CTL at the time of the cytotoxicity assays. The possibility of HLA-

Table 3
Percentages of IFN-γ- and IL-4-positive $CD8^+$ T cells from PBL and TIL derived from cervical cancer patients

	Patient 1				Patient 2				Patient 3			
	Before		After		Before		After		Before		After	
	IFN-γ	IL-4	IFN-γ	IL-4	IFN-γ	IL-4	IFN-γ	IL-4	IFN-γ	IL-4	IFN-γ	IL-4
PBL	12	3	48	11	20	4	49	12	14	3	61	19
TIL	63	0.5	78	4	59	1	89	3	56	0.5	65	7

Percentages of IFN-γ- and IL-4-positive $CD8^+$ T cells from PBL and TIL before and after in vitro stimulation with E7-pulsed autologous dendritic cells. The difference in IFN-γ expression in PBL versus TIL before and after DC stimulation was significant at $P > 0.05$. The difference in IL-4 expression in PBL versus TIL before and after DC stimulation was significant at $P > 0.05$.

unrestricted lysis of target cells by at least some tumor-specific $CD8^+$ T cells cannot be excluded [42]. Indeed, high-avidity/high-affinity tumor-specific $CD8^+$ CTL with the ability to kill tumor target cells using either HLA class-I-restricted or a HLA class-I-unrestricted mechanisms have been previously reported [42].

Lymphocytes used at the time of in vitro stimulation with HPV16 or HPV18 E7-loaded DC were predominantly $CD4^+$ T cells in PBL and $CD8^+$ T cells in TIL (CD4:CD8 ratios, 2:1 and 0.8:1, respectively). After in vitro restimulation with antigen-loaded fully mature DC, $CD4^+$ T cells remained predominant in PBL cultures, while in TIL cultures a predominant population of $CD8^+$ T cells was again consistently noted. $CD56^+/CD16^+/CD3^-$ NK cells and $CD8^+/CD56^+$ T cells were predominant in PBL compared to TIL at the beginning of culture. However, after in vitro stimulation and expansion of $CD8^+$ T cells, TIL contained a significantly higher percentage of $CD8^+/CD56^+$ T cells compared to PBL. In agreement with previous studies [43,44], the percentage of $CD8^+/CD56^+$ T cells in the PBL and TIL cultures was found to correlate with the overall cytotoxic activity.

TIL derived from cervical cancer specimens have been reported to have reduced expression of CD3ζ, an important signaling component of the T cell receptor [45]. Importanty, T cells with suppressed ζ chain have been demonstrated to exhibit diminished proliferation and production of cytokines [45]. In this study, consistent with this previous report [45], we found a lower relative expression of TcR ζ chain in freshly isolated $CD8^+$ T cells derived from TIL compared to PBL. However, no significant difference in TcR ζ chain expression compared to PBL was detected after in vitro stimulation of TIL with HPV16 and HPV18 E7-pulsed fully mature DC. These data, therefore, demonstrate that fully mature DC stimulation may provide culture conditions able to induce restoration of TcR ζ chain expression in TIL to the level detected in activated PBL populations.

Induction of in vivo antitumor immune responses and effective adoptive cellular immunotherapy have been reported to be more dependent on the induction of a host immune response triggered by Type 1 cytokines (i.e., IL-2, IFN-γ, GM-CSF) than the in vitro cytotoxic activity of the responder or transferred lymphocytes [46–48]. In support of this view, studies of Tartour et al. [10] have recently shown that poor prognosis and tumor recurrence in cervical cancer patients was associated with detection of low numbers of IFN-γ mRNA copies in fresh tumor biopsy specimens. Furthermore, a significant dysfunction of Type 1 T cell responses concomitant with an abnormally elevated production of Type 2 cytokines in cervical cancer patients has been reported [49], suggesting that tumor progression may be associated with a preferential and unprotective Type 2 T cell response. In our previous work comparing the phenotypes of lymphocytes derived from PBL and TIL in invasive cervical cancer patients [36], IFN-γ expression was predominant in the $CD8^+$ T cell populations derived from TIL. Consistent with these data, in all three patients studied in this report, IFN-γ expression was predominant in TIL compared to the PBL-derived $CD8^+$ T cell populations $IL\text{-}4^+$ T cells were detected in low numbers and mainly in PBL. After in vitro stimulation with tumor antigen-pulsed DC, a greater proportion of $IFN\text{-}\gamma^+$ Th1 cells was again detected in TIL compared to PBL. This pattern of cytokine expression in TIL, combined with the consistently inverted CD4 to CD8 ratios, further supports the concept of a specific recruitment and accumulation of a high concentration of antigen-experienced tumor-specific $CD8^+$ T lymphocytes in the tumor tissue [36,50].

In conclusion, we showed that TIL may constitute a superior source of tumor-specific HLA class-I-restricted CTL compared to PBL. Although poor immune responses by explanted T lymphocytes might be expected, it is possible to normalize defective TIL responses by in vitro T cell stimulation with professional antigen presenting cells, such as fully mature DC. These results, combined with the fact that the most critical component of successful adoptive immunotherapy of cancer is the identification and isolation of large numbers of lymphocytes with potent and specific antitumor activity, support further research efforts for the development of improved protocols to counteract mechanisms leading to impaired TIL function and thus exploit these cells as potential lymphocyte populations for use in adoptive cellular immunotherapy of cervical cancer patients.

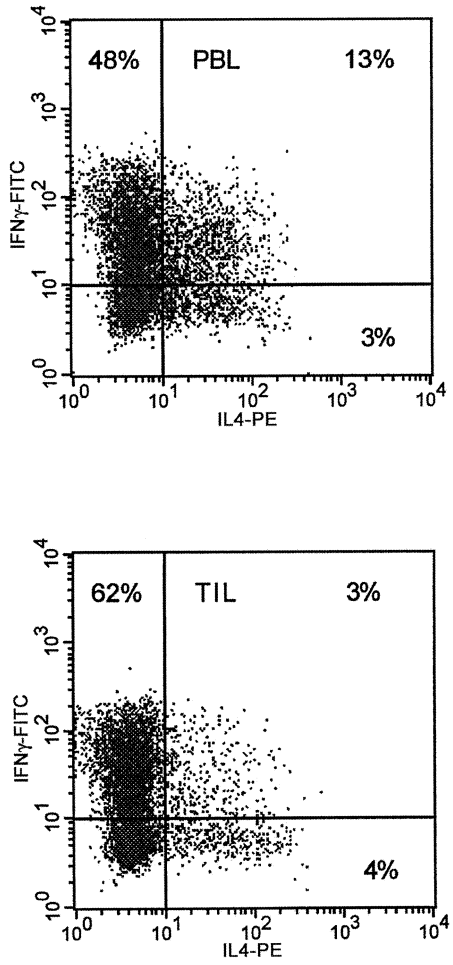

Fig. 3. Representative dot plot analysis of intracellular IFN-γ versus IL-4 expression by $CD8^+$ T cells from an individual patient after in vitro culture with full-length HPV16/18 E7-loaded DC. Lymphocytes were activated by PMA and ionomycin as described under Materials and Methods. Numbers in the quadrants represent the percentages of cytokine-positive $CD8^+$ T lymphocytes.

Acknowledgments

This work was supported in part by grants from the Camillo Golgi Foundation, Brescia, Italy, and the Lega Nazionale contro i Tumori Sezione di Brescia.

References

[1] DiSaia PJ, Creasman WT. Cervical cancer. In: DiSaia PJ, Creasman WT, editors. Clinical gynecologic oncology. St. Louis: Mosby–Year Book, Inc., 1997. p. 1–106.

[2] Brinton LA. Epidemiology of cervical cancer: Overview. In: Munoz N, Bosh F, Shah KV, Meheus A, editors. The epidemiology of human papillomavirus and cervical cancer, Vol. 119. Lyon, France: IARC Science Publications, 1992. p. 3–23.

[3] Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R, Shah KV. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. J Natl Cancer Inst 1995;87:796–802.

[4] Penn I. Cancers of the anogenital region in renal transplant recipients: analysis of 65 cases. Cancer 1988;53:539–45.

[5] Rellihan MA, Dooley DP, Burke TW, Berkland ME, Longfield RN. Rapidly progressing cervical cancer in a patient with human immunodeficiency virus infection. Gynecol Oncol 1990;36:435–8.

[6] Nasiell K, Roger V, Nasiell M. Behaviour of mild cervical dysplasia during long term follow-up. Obstet Gynecol 1986;67:665–9.

[7] Rogozinski T, Jablonska S, Jarzabek-Chorzelska M. Role of cell-mediated immunity in spontaneous regression of plane warts. Int J Dermatol 1988;27:322–8.

[8] Morison WL. Viral warts, herpes simplex, and herpes zoster in patients with secondary immune deficienses and neoplasms. Br J Dermatol 1975;92:625–33.

[9] Chao HT, Wang PH, Tseng JY, Lai CR, Chiang SC, Yuan CC. Lymphocyte-infiltrated FIGO stage IIB squamous cell carcinoma of the cervix is a prominent factor for disease-free survival. Eur J Gynaec Oncol XX 1998;2:136–40.

[10] Tartour E, Gey A, Sastre-Garau E, Lombard Surin I, Mosseri V, Fridman WH. Prognostic value of intratumoral interferon gamma messanger RNA expression in invasive cervical carcinomas. J Natl Cancer Inst 1998;90:287–94.

[11] Higgins GD, Davy M, Roder D, Uzelin DM, Phillips GE, Burrel CJ. Increased age and mortality associated with cervical carcinomas negative for human papillomavirus RNA. Lancet 1991;338:910–13.

[12] Meneguzzi G, Cerni C, Kieny MP, Lathe R. Immunization against human papilloma virus type 16 tumor cells with recombinant vaccinia viruses expressing E6 and E7. Virology 1991;181:62–9.

[13] Chen L, Thomas EK, Hu S-L, Hellstrom I, Hellstrom KE. Human papilloma virus type 16 nucleoprotein E7 is a tumor rejection antigen. Proc Natl Acad Sci USA 1991;88:110–14.

[14] Chen L, Mizuno MT, Singal MC, Hu S-L, Galloway DA, Hellstrom I, Hellstrom KE. Induction of cytotoxic T lymphocytes specific for a syngeneic tumor expressing the E6 oncoprotein of human papilloma virus type 16. J Immunol 1992;148:2617–21.

[15] Alexander M, Salgaller ML, Celis E, Sette A, Barnes WA, Rosenberg SA, Steller MA. Generation of tumor-specific cytolytic T lymphocytes from peripheral blood of cervical cancer patients by in vitro stimulation with a synthetic human papillomavirus type 16 E7 epitope. Am J Obstet Gynecol 1996;175:1586–93.

[16] Evans EM, Man S, Evans AS, Borysiewicz LK. Infiltration of cervical cancer tissue with human papillomavirus-specific cytotoxic T-lymphocytes. Cancer Res 1997;57:2943–50.

[17] Nimaco M, Fiander AN, Wilkinson GWG, Borysiewicz LK, Man S. Human papillomavirus-specific cytotoxic T lymphocytes in patients with cervical intraepithelial neoplasia grade III. Cancer Res 1997;57:4855–61.

[18] Borysiewicz LK, Fiander A, Nimako M, Man S, Wilkinson GW, Westmoreland D, Evans AS, Adams M, Stacey SN, Boursnell ME, Rutherford E, Hickling JK, Inglis SC. A recombinant vaccinia virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. Lancet 1996;347:1523–7.

[19] Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 1996;9:271–96.

[20] Romani N, Gruner S, Brang D, Kampgen E, Lenz A, Trockenbacher B, Konwalinka GO, Fritsch P, Steinman RM, Shuler G. Proliferating dendritic cell progenitors in human blood. J Exp Med 1994;180:83–93.

[21] Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony stimulating factor plus interleukin-4 and down-regulated by tumor necrosis factor α. J Exp Med 1994;179:1109–16.

[22] Hsu FJ, Benike C, Fagnoni F, Liles TM, Czerwinski D, Taidi B, Engleman EG, Levy R. Vaccination of patients with B-cell lymphoma using autologous antige-pulsed dendritic cells. Nature Med 1996;2:52–8.

[23] Nestle FO, Alijagic S, Gilliet M, Sun Y, Grabbe S, Dummer R, Burg G, Schadendorf D. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nature Med 1998;4:328–32.
[24] Thurner B, Haendle I, Roder C, Dieckmann D, Keikavoussi P, Jonuleit H, Bender A, Maczek C, Schreiner D, von den Driesch P, Brocker EB, Steinman RM, Enk A, Kampgen E, Schuler G. Vaccination with Mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J Exp Med 1999;190:1669–78.
[25] Kugler A, Stuhler G, Walden P, Zoller G, Zobiwalski A, Brossart P, Trefzer U, Ullrich S, Muller CA, Becker V, Gross AJ, Hemmerlein B, Kanz L, Muller GA, Ringert R-H. Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cells hybrid. Nature Med 2000;6:332–6.
[26] Geiger JD, Hutchinson RJ, Hohenkirk LF, McKenna EA, Yanik GA, Levine JE, Chang AE, Braun TM, Mule JJ. Vaccination of pediatric solid tumor patients with tumor lysate-pulsed dendritic cells can expand specific T cells and mediate tumor regression. Cancer Res 2001;61:8513–19.
[27] Rosenberg SA, Spiess P, Lafreniere R. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. Science 1987;233:1318–22.
[28] Spiess P, Yang JC, Rosenberg SA. In vivo antitumor activity of tumor infiltrating lymphocytes expanded in recombinant interleukin-2. J Natl Cancer Inst 1987;79:1067–73.
[29] Ioannides CG, Whiteside TL. T cell recognition of human tumors: implications for molecular immunotherapy of cancer. Clin Immunol Immunopathol 1993;66:91–106.
[30] Whiteside TL. Tumor infiltrating lymphocytes in human malignancies. Austin TX: Medical Intelligence Unit, R.G. Landes; 1993.
[31] Tartour E, Latour S, Mathiot C, Thiounn N, Mosseri V, Joyeux I, Dubois D'Enghien C, Lee R, Debre B, Fridman WH. Variable expression of CD3-ζ chain in tumor infiltrating lymphocytes (TIL) derived from renal-cell carcinoma: relationship with TIL phenotype and function. Int J Cancer 1995;63:205–12.
[32] Renner C, Ohnesorge S, Held G, Bauer S, Jung W, Pfitzenmeier J-P, Pfreundschuh A. T cells from patients with Hodgkin's disease have a defective T-cell receptor ζ chain expression that is reversible by T-cell stimulation with CD3 and CD28. Blood 1996;88:236–41.
[33] Santin AD, Hermonat PL, Ravaggi A, Chiriva-Internati M, Cannon M, Hiserodt JC, Pecorelli S, Parham GP. Expression of surface antigens during the differentiation of human dendritic cell versus macrophages from blood monocytes in vitro. Immunobiology 1999; 200:187–204.
[34] Santin AD, Hermonat PL, Ravaggi A, Chiriva-Internati M, Zhan DJ, Pecorelli S, Parham GP, Cannon M. Induction and characterization of human papillomavirus-specific cytotoxic T lymphocytes by E7 pulsed autologous dendritic cells in patients with HPV-16 and 18 positive cervical cancer. J Virol 1999;3:5402–10.
[35] Whiteside TL, Miescher S, MacDonald HR, Von Fliedner V. Separation of tumor infiltrating lymphocytes from tumor cells in human solid tumors. A comparison between velocity sedimentation and discontinuous density gradient. J Immunol Methods 1986;90:221–33.
[36] Santin AD, Ravaggi A, Bellone S, Pecorelli S, Cannon M, Parham GP, Hermonat P. Tumor infiltrating lymphocytes contain higher numbers of type 1 cytokine expressors and DR+ T cells compared with lymphocytes from tumor draining lymph nodes and peripheral blood in patients with cancer of the uterine cervix. Gynecol Oncol 2001; 81:424–32.
[37] Santin AD, Hermonat PL, Ravaggi A, Cannon MJ, Pecorelli S, Parham GP. Secretion of vascular endothelial growth factor in adenocarcinoma and squamous cell carcinoma of uterine cervix. Obstet Gynecol 1999;94:78–82.
[38] Melichar B, Savary C, Kudelka AA, Verscharaegen C, Kavanagh JJ, Edwards CL, Platsoucas CD, Freedman RL. Lineage-negative human leukocyte antigen-DR+ cells with the phenotype of undifferentiated dendritic cells in patients with carcinoma of the abdomen and pelvis. Clin Cancer Res 1998;4:799–809.
[39] Onsurd M, Thorsby E. Long term changes in natural killer cytotoxicity after external pelvic radiotherapy. Int J Radiat Oncol Biol Phys 1981;7:609–14.
[40] Balaram P, Pillai R, Padmanabhan TK, Abraham T, Hareendran NK, Nair MK. Immune function in malignant cervical neoplasia: A multiparameter analysis. Gynecol Oncol 1988;31:409–23.
[41] Santin AD, Hermonat PL, Ravaggi A, Bellone S, Roman J, Pecorelli S, Cannon M, Parham GP. Effects of concurrent cisplatinum administration during radiotherapy versus radiotherapy alone on the immune function of patients with cancer of the uterine cervix. Int J Radiat Oncol Biol Phys 2000;48:997–1006.
[42] Roberts TE, Shipton U, Moore M. Role of MHC class I antigens and the CD3 complex in the lysis of autologous human tumors by T cell clones. Int J Cancer 1987;39:436–41.
[43] Santin AD, Hermonat PL, Ravaggi A, Bellone S, Roman JJ, Jayaprabhu S, Pecorelli S, Parham GP, Cannon M. Expression of CD56 by human papilomavirus E7-specific $CD8^+$ cytotoxic T lymphpcytes correlates with increased intracellular perforin expression and enhanced cytotoxicity against HLA A2-matched cervical tumor cells. Clin Cancer Res 2001;7:804s–10s.
[44] Hilders CGJM, Ras L, Van Eendenburg JDH, Nooyen Y, Fleuren GJ. Isolation and characterization of tumor-infiltrating lymphocytes from cervical carcinoma. Int J Cancer 1994;57:805–13.
[45] Kono K, Ressing ME, Brandt RMP, Melief JM, Potkul RK, Andersson B, Petersson M, Kast WM, Kiessling R. Decreased expression of signal-transducing ζ chain in peripheral T cells and natural killer cells in patients with cervical cancer. Clin Cancer Res 1996;2:1825–8.
[46] Romagnani S. Human TH1 and TH2 subsets: regulation of differentiation and role in protection and immunopathology. Int Arch Allergy Immunol 1992;98:279–85.
[47] Barth RJJ, Mule JJ, Spiess PJ, Rosenberg SA. Interferon gamma and tumor necrosis factor have a role in tumor regression mediated by murine $CD8^+$ tumor infiltrating lymphocytes. J Exp Med 1991;173: 647–54.
[48] Goedegebuure PS, Zuber M, Leonard-Vidal DL, Burger UL, Cusack JC, Chang MP, Douville LM, Eberlein TJ. Reactivation of murine tumor-infiltrating lymphocytes with solid phase anti-CD3 antibody: in vitro cytokine production is associated with in vivo efficacy. Surg Oncol 1994;3:79–89.
[49] Clerici M, Shearer GM, Clerici E. Cytokine disregulation in invasive cervical carcinoma and other human neoplasias: time to consider the Th1/Th2 paradigm. J Natl Cancer Inst 1998;90:261–2.
[50] Yamamoto K, Masuko K, Takahashi S, Ikeda Y, Kato T, Mizushima Y, Hayashi K, Nishioka K. Accumulation of distinct T cell clonotypes in human solid tumors. J Immunol 1995;154:1804–9.