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Increase of human papillomavirus-16 E7-specific T helper type 1 response in peripheral blood of cervical cancer patients after radiotherapy

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Summary

It has been suggested that tumour cell lysis by gamma-radiation induces a tumoral antigen release eliciting an immune response. It is not clear how a specific immune response in cervical cancer patients is developed after radiotherapy. This study is an attempt to investigate the role of the human papillomavirus type 16 (HPV-16) E7-specific T helper response before and after radiotherapy. Lymphocytes were isolated from 32 cervical cancer patients before and after radiotherapy and from 16 healthy women. They were stimulated for 12 hr with autologous HPV-16 E7-pulsed monocyte-derived dendritic cells or directly with HPV-16 E7 synthetic peptides: $E7_{51–70}$, $E7_{65–84}$ and $E7_{79–98}$. The cells were stained for CD4, CD69, intracellular interferon-γ (IFN-γ) and interleukin-4 (IL-4) cytokines and analysed by flow cytometry. A specific $CD4^+$ $CD69^+$ IFN-γ^+ immune response against HPV-16 $E7_{79–98}$ peptide was observed in 10 of 14 patients (71·4%) after treatment, compared with 4 of 14 (28·5%) before radiotherapy ($P = 0·039$); however, this response was not associated with a successful clinical response. Before treatment, 5 of 31 patients showed a HPV-16 $E7_{79–98}$-specific T helper type 2 (Th2) response. Interestingly, this response was significantly associated with a decrease in disease-free survival ($P = 0·027$). These results suggest that a Th2-type cellular response could be useful as a predictor of recurrence and poor prognosis. An increase of the HPV-specific immune response was observed after radiotherapy; however, it is not enough to control completely the disease after treatment. Our results support that the E7-specific T-cell IFN-γ response in cervical cancer patients, rather than reflecting the host's capability of controlling tumour growth, might be an indicator for disease severity.

Keywords: cervical cancer; T helper-inducer; human leucocyte antigen class I; human papillomavirus 16 E7 protein; radiotherapy; T lymphocytes

Introduction

Several epidemiological studies have established that infection with high-risk human papillomavirus (HPV) genotypes represents the most important risk factor in the development of cervical neoplasias.[1–3] The HPV-16 E6 and E7 genes are frequently coexpressed in tumour cells and they are the most abundant viral transcripts found in HPV-16-positive cervical cancer biopsies.[4] The E6 and E7 oncoproteins are therefore attractive targets for T-cell-mediated immunotherapy.[5–7]

There is evidence that a cell-mediated immune response plays an important role in the control of both HPV infections and HPV-associated neoplasm. It has been deduced by the higher incidence of associated genital cancer and the rapid progression of HPV-associated tumours in both immunosuppressed transplanted patients and individuals with human immunodeficiency virus infection.[8,9] In addition, specific cytotoxic T-cell and T helper activity to E6 and E7 from HPV-16 and HPV-18 have been demonstrated in peripheral blood from patients with premalignant cervical neoplasias, and it has been suggested that spontaneous regression of cervical intraepithelial neoplasia lesions might be associated with these immune responses.[10–12]

Analysis of T helper type 1 (Th1) and type 2 (Th2) cytokine profiles has been used to characterize the

immune response in several human diseases, including HPV-associated diseases.[13,14] Some of these studies have shown that, during carcinogenesis in the cervical epithelia, a shift from Th1 to Th2 cytokines is observed.[14,15] In contrast, other studies have suggested that cervical cancer progression is associated with specific immune failure in response to HPV-16 and that it is not related to a shift to a Th2 cytokine profile.[13]

Radiotherapy is the most important non-surgical treatment for cervical carcinoma.[16] Over the last 20 years, advances in radiotherapy and the use of adjuvant chemotherapy have improved the treatment of patients with advanced stages of cervical cancer. In addition, an important factor in radiotherapy eliminating tumour cells seems to be the induction of an antitumoral immune response. Experimental models suggest that lysis of tumour cells by gamma-radiation could increase tumoral antigen release, reactivating the tumoral immune response.[17,18] It has also been observed that ablation of tumour cells by radiofrequency can induce immunostimulatory signals that activate antigen-presenting cells and so induce an immune response. This response can protect some mice when they are challenged with a second lethal dose of living tumour cells.[17] This evidence suggests that even procedures commonly used in clinical practice, which result in the synchronized death of tumour cells, have the potential of initiating a specific immune response.

At present, the characteristics of the E7-specific cellular immune response in women with cervical cancer after receiving radiotherapy are not well understood, and there is no evidence of whether an increase in the HPV-specific immune response after treatment contributes to the treatment's success. Therefore, the aim of this study was to evaluate the effect of radiotherapy, with or without concurrent cisplatin, on HPV-16 E7-specific immune response in patients with advanced cervical cancer. For this purpose, the frequencies of HPV-16 E7-specific T-helper cells producing interferon-γ (IFN-γ) or interleukin-4 (IL-4) were detected in women with cervical cancer before and after treatment and in women with normal cytology.

Materials and methods

Patients and controls

Thirty-two HPV-16-positive patients with invasive cervical cancer staged IBI to IIIB according to FIGO (International Federation of Gynecologist and Obstetricians) attending the outpatients gynaecological clinic of the Instituto Nacional de Cancerología (INC), in Bogotá (Colombia), from October 2004 to October 2006, were enrolled in the study. Patients were not included if they had undergone any treatment before radiotherapy or if they showed prior or concurrent second malignancies. A group of 16 non-pregnant women with normal cervical cytology and no immune disorders, who attended the Liga de Lucha contra el Cancer, also in Bogotá, were included as a control group. This study was approved by the INC Medical Ethics Committee, and informed consent was obtained from patients and controls.

During the first gynaecological examination, cervical scrapes were collected from each patient using a spatula and brush and placed in tubes containing 5 ml phosphate-buffered saline (PBS 1 ×) and 0·05% thiomerosal to determine the presence of HPV DNA. At a second visit, 60 ml heparinized venous blood was obtained from HPV-16-positive patients before treatment, and at a third visit 3 months after completion of radiotherapy another blood sample was taken. For the control group, cervical scrapes and blood samples were taken during the same gynaecological visit.

Cervical cancer patient treatment

All patients underwent primary radiotherapy, with or without concurrent cisplatin, according to the radiotherapy protocols of the INC.[19] Briefly, external beam radiotherapy was delivered homogeneously to the pelvis using a planned dose of 45–50·4 Gy in 25 to 28 fractions of 180 cGy employing the box technique. In addition, intracavitary high or low dose-rate brachytherapy was applied depending on tumour stage and volume. Patients who received chemotherapy were treated weekly with 40 mg/m^2 cisplatin concurrently with external beam therapy during 6 weeks.

HPV testing

Cells from cervical scrapes were detached from the spatula and brush by vortex and subsequently centrifuged at 3000 ***g*** for 10 min. The cell pellet was suspended in 1 ml buffered Tris–HCl 10 mM pH 8·3 and stored at − 70° until use. To assess the quality of the target DNA all pellets were prescreened using a 209 base pair amplifying β-globin polymerase chain reaction (PCR) using PCO3 and PCO5 primers as described by Molano *et al.* 2002.[20] The HPV typing was performed by PCR using generic primers GP5$^+$/GP6$^+$.[3] The specific detection of HPV-16 was carried out by PCR amplifying a 561 base pair segment of the E6 gene. The sequences of primers used were 5′-AAACTAAGGGCG TAACCG-3′ and 5′-TGTAGGTGTATCTCCATGC-3′. As a positive control, SiHa DNA cells were used.

Antibodies and reagents

The cytometry analysis used the following monoclonal antibodies (mAbs): anti-CD14 conjugated with fluorescein isothiocyanate (FITC; M5E2), anti-CD1a conjugated with phycoerythrin (PE; HI149), anti-CD83 FITC (HB15e), anti-HLA-DR PE (G46-6), anti-CD86 FITC (2331), anti-CD69 conjugated with allophycocyanin (L78), anti-CD4

conjugated with peridinin chlorophyll protein (SK3), anti-IFN-γ FITC (4S.B3), anti-IL-4 PE (8D4-8) and isotype controls FITC and PE (MOPC-21 and UPC-10) (all purchased from BD Biosciences, San Diego, CA and San Jose, CA). The recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF) was purchased from BD. Recombinant human IL-4 and lipopolysaccharide (LPS; *Escherichia coli* 026:B6) were purchased from SIGMA (St Louis, MO).

Antigens

Recombinant HPV-16 E7 protein was produced and purified as follows: *Escherichia coli* JM101 transformed with pMAL-HPV-16 E7 plasmid was cultivated in Lauria-Bertoni (LB) medium with stirring at 37° overnight. The culture was then diluted 1/10 in LB medium and grown for 1·5 hr at 37°. Then, 0·1 mM isopropyl-1-thio-ß-D-galactoside (IPTG) was added and culture was continued for 4 hr. The MBP-HPV-16 E7 fusion protein was purified following the instruction manual of pMAL™ Protein Fusion and Purification System (BioLabs New England Inc., Beverly, MA). The HPV-16 E7 protein was cleaved by Factor Xa. The maltose-binding protein (MBP) purified was used as a negative control. The sodium dodecyl sulphate–polyacrylamide gel electrophoresis (12·5%) analysis showed an approximately 21 000 protein with more than 90% purity. This protein was identified as HPV-16 E7 by Western blot by using a specific antibody against HPV-16 E7 protein from serum of a patient with cervical cancer that had been previously tested.[2] The endotoxin level in the HPV-16 E7 preparations was very low (3.5×10^{-4} EU/ml) as determined by a *Limulus* amoebocyte lysate assay (Bio Whittaker Inc., Walkersville, MD) following the manufacturer's instructions. Additionally, three 20-residue long overlapping (by five residues) synthetic peptides from the C-terminal region of HPV-16 E7 were purchased from Invitrogen Life Technologies (Carlsbad, CA). The sequences of the peptides were as follows: $E7_{51–70}$: HYNIVTFCCKCDST-LRLCVQ; $E7_{65–84}$: LRLCVQSTHVDIRTLEDLLM; and $E7_{79–98}$: LEDLLMGTLGIVCPICSQKP. Each peptide was dissolved at 1 mg/ml in PBS containing 10% of dimethyl sulphoxide (DMSO) and stored at −20°. As a negative control, a peptide (20-residue long) from MSA-2 (merozoite surface antigen 2 of *Plasmodium falciparum*) protein was used: KNESKISNTFINNAYNMSIR.

Peripheral blood mononuclear cell isolation and dendritic cell generation

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood by Ficoll–Hypaque density gradient centrifugation (SIGMA) and used either for dendritic cell (DC) generation or peptide stimulation. The monocytes were separated from PBMCs by negative selection using magnetic-activated cell sorting (MACS) beads and MACS® separation columns (MACS®, Miltenyi Biotech, Bergisch Gladbach, Germany) following the manufacturer's instructions. Monocyte purity was determined by flow cytometry analysis of CD14 expression that must be between 80% and 90% of the total cells. The monocyte-depleted PBMCs were cryopreserved in fetal bovine serum (FBS, GIBCO, Grand Island, NY) containing 10% dimethyl sulphoxide until its use.

The DCs were obtained by *in vitro* differentiation of monocytes as described by Sallusto and Lanzavecchia.[21] Monocytes were cultured in RPMI-1640 supplemented with 1000 U/ml rhGM-CSF (BD Biosciences), 200 U/ml rhIL-4 (SIGMA) and 10% of FBS (GIBCO) at 37° in 5% CO_2 and 95% humidity for 5 days. Fresh medium supplemented with cytokines was added to the cultures on day 3. On day 5, non-adherent cells corresponding to immature DCs (iDCs) were harvested and used for antigen loading. The endotoxin levels from media and all reagents used to obtain DCs were below 0·03 EU/ml.

Protein-pulsed monocyte-derived mature dendritic cells

Immature DCs were harvested and distributed on 24-well plates at a density of $1·5 \times 10^5$ to 3×10^5 cells/well, pulsed with 10 μg/ml HPV-16 E7 protein or 10 μg/ml MBP or incubated with 100 ng/ml of LPS in RPMI-1640 containing 10% FBS, and incubated for 2, 6, 10, 24 and 48 hr. To obtain an optimal DC maturation, 2 hr after HPV-16 E7 protein and MBP addition, iDCs were incubated with 100 ng/ml LPS for 24 hr or 48 hr. The monocyte-derived DCs were analysed by flow cytometry for the expression of CD1a, CD86, HLA-DR and CD83. To confirm uptake of HPV-16 E7 protein by DC, the iDCs were transferred onto slides at a density of 3×10^4 iDC/well, fixed with 2% paraformaldehyde solution and tested by indirect immunofluorescence. The fixed cells were briefly washed with PBS 1 ×, and unspecific binding sites were blocked with 5% horse serum in PBS for 30 min at room temperature. Subsequently, the slides were stained with 20 μl of a 1/10 serum dilution from a patient with cervical cancer and positive for HPV-16 E7 antibodies.[2] After three washes, the slides were incubated with 10 μl of a 1/100 dilution of human anti-immunoglobulin G (IgG) antibody conjugated with FITC (Vector Laboratories Inc., Burlingame, CA). After three further washes, the slides were stained with Evans Blue. Finally, the slides were analysed by fluorescence microscopy (Olympus provis AX70; Olympus Americana Inc., Woodbury, NY) with interferometer and image analyser (Applied Spectral Imaging, Haifa, Israel).

Lymphocyte stimulation with E7-pulsed autologous DCs

Cryopreserved monocyte-depleted PBMCs were thawed, washed once with PBS, and suspended in an AIM-V

medium supplemented with 10% FBS at $1{\cdot}5 \times 10^6$ cells/well in 24-well culture plates and cocultivated with E7-pulsed or MBP-pulsed autologous DCs (ratios from 5 : 1 to 10 : 1) for 12 hr. As a positive control, 1·25 μg/ml Staphylococcal enterotoxin B (SEB; Sigma) was added to LPS-stimulated autologous DC cocultures, and LPS-stimulated autologous DC cocultures without antigen were used as a negative control. Between 8 and 10 hr before harvesting the cells for analysis, 10 μg/ml brefeldin A (Sigma) was added.

PBMC stimulation with HPV-16 E7 peptides

The PBMCs were suspended in an AIM-V medium supplemented with 10% FBS at $1{\cdot}5 \times 10^6$ cells/well in 24-well culture plates and stimulated separately with 10 μg/ml of each HPV-16 E7 peptide or MSA-2 peptide for 12 hr. As a positive control, the PBMCs were stimulated with SEB (1·25 μg/ml), and as a negative control the PBMCs were cultured in medium alone. Between 8 and 10 hr before harvesting the cells for analysis, 10 μg/ml brefeldin A was added.

Flow cytometry analysis of surface antigens and intracellular cytokines

After stimulation with each antigen, lymphocytes were harvested and stained for superficial antigen expression by using directly conjugated mAbs anti-CD4 and anti-CD69 for 30 min at 4°. For analysis of intracellular cytokine expression, the cells were washed with PBS and fixed and treated with Cytofix/Cytoperm (BD Biosciences) for 20 min at 4°. Then the cells were washed once with Perm/Wash 1× (Pharmingen) and incubated with anti-IFN-γ and anti-IL-4 for 30 min at 4° and finally washed twice with Perm/Wash 1× and suspended in 200 μl PBS–paraformaldehide 0·05% solution. Control cells were stained with the respective isotype controls. All mAbs were previously titrated, and optimal concentrations were used. The analysis of stained cells was performed using a four-colour FACSCalibur™ flow cytometer (BD Immunocytometry Systems, San Jose, CA). Between 30 000 and 50 000 gated $CD4^+$ events were acquired and analysed using CELL QUEST PRO™ software. Although the mean frequency background in the unstimulated cultures was < 0·02 for both PBMCs stimulated with medium alone and PBMCs stimulated with MBP-pulsed DCs, we decided to calculate net frequencies.

HPV virus-like particle ELISA

The HPV virus-like particles (VLPs) were produced in insect cells using recombinant baculovirus encoding HPV-16 L1 protein and purified as described by Combita *et al.*[22] Protein concentration was determined by MicroBSA assay (Bio-Rad Laboratories, Hercules, CA). The VLPs were added at the optimum concentration, which was determined using pools of positive control sera. Ninety-six-well microplates (Nunc, Life Technologies, Rochester, NY) were coated overnight at 4° with 200 ng HPV-16 L1 VLPs or 200 ng bovine serum albumin (BSA) in PBS, pH 7·4. Each serum sample and BSA was tested twice at the same plate. After washing with PBS, 200 μl PBS containing 5% FBS and 0·1% Tween-20 were added for 2 hr at 37°. The blocking solution was replaced by 100 μl sera diluted 1 : 20 in 5 × PBS, 10% FBS and 2% Tween-20, and plates were incubated at 45° for 60 min. After five washes, bound antibodies were detected with a horseradish peroxidase-conjugated goat anti-human IgG immunoglobulin (diluted 1 : 5000; Sigma). Following incubation at 45° for 1 hr and four washes, 100 μl of a substrate solution containing 0·1% 2,2′-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS) in 50 mM citrate/phosphate buffer and 0·03% hydrogen peroxide were added. After 30 min incubation, the optical density (OD) was determined at 405 nM with an automated plate reader (BioRad, model 550). For each serum sample the background reactivity found in the BSA-coated wells was subtracted from the OD found in each of the HPV-VLP-coated wells. Negative values were adjusted to zero. The cut-off was calculated from 70 sera of women with normal cytology who were DNA HPV and HPV-VLP ELISA negative. This value was 0·216.

Human leucocyte antigen class I immunohistochemistry

Eighteen of 32 biopsies from women with cervical cancer were analysed for human leucocyte antigen (HLA) expression. Four-micrometre-thick cryostat sections were mounted on glass slides, air-dried overnight, fixed with precooled acetone for 10 min, and stored at −20° until used. Endogenous peroxidase activity was blocked with 0·3% H_2O_2 for 15 min and non-specific binding sites were blocked by incubating slides with 20% AB serum/PBS for 20 min at room temperature. Then the slides were incubated for 30 min at room temperature with 1 : 100 mouse anti-HLA I mAb (W6/32, BD Pharmingen, San Diego, CA). As a negative control, PBS was used. A secondary biotinylated goat antibody to mouse immunoglobulin (DakoCytomation Denmark A/S, Glostrup, Denmark) was applied and incubated at room temperature for 30 min. The slides were then incubated with an avidin–biotin–peroxidase conjugate for 30 min. The immunohistochemical reactions were developed with a fresh 3,3′-diaminobenzidine tetrahydrochloride solution. The slides were counterstained with haematoxylin, dehydrated through alcohol, and cleared in xylene before mounting.

Statistical interpretation

The net frequencies of $CD4^+$ $CD69^+$ IFN-γ^+ or $CD4^+$ $CD69^+$ IL-4^+ T cells responding to HPV-16 E7

producing IFN-γ or IL-4 in responders were obtained by subtracting the percentage of T helper cells responding to the negative control (without antigen) from the percentage of T helper cells responding to the viral antigen. The cut-off level over which frequencies were considered positive was based on the distribution of frequency values observed in DNA HPV-negative and VLP HPV-negative control women with normal cytology. This cut-off value was calculated by adding three times the standard deviation to the mean frequencies for each antigen. The cut-off values for IFN-γ responses were 0·025, 0·029, 0·025 and 0·020 for $E7_{51–70}$ peptide, $E7_{65–84}$ peptide, $E7_{79–98}$ peptide and E7protein, respectively. For IL-4 responses the cut-off values were 0·023, 0·015, 0·018 and 0·026 for $E7_{51–70}$ peptide, $E7_{65–84}$ peptide, $E7_{79–98}$ peptide and E7protein, respectively. The frequencies are shown as mean ± SEM. The non-parametric Mann–Whitney *U*-test was used to evaluate differences in mean frequencies between groups. The association between HPV-16 E7-specific T helper response and the patients' prognosis was determined. The survival rates were calculated by the Kaplan–Meier method and the differences between the survival curves were determined by the log-rank test. For all tests, $P < 0·05$ was considered statistically significant. Statistical analysis was performed using SPSS 15.0 software. Disease-free survival (DFS) was defined as the interval from the end of the treatment to clinically or radiologically proven recurrence and overall survival (OS) was defined as the interval from beginning of the treatment to death or last visit date.

Results

Clinical characteristics of the patients

The clinical characteristics of the study subjects are listed in Table 1. A total of 32 HPV-16-positive women with a diagnosis of invasive cervical cancer stage IBI ($n = 3$), IIA ($n = 2$), IIB ($n = 9$) or IIIB ($n = 18$) were enrolled in the study. The age range was 27–62 years. Twenty-one patients (67·2%) received a combined chemotherapy and radiotherapy, and 10 patients (31·2%) received radiotherapy alone. Only one patient did not accept any therapy. Tumour sizes before treatment ranged from 1·5 cm to 7·25 cm. Twenty-eight of 31 patients were followed after treatment. The mean follow-up time was 24·1 months, with a time range from 1·8 to 41·9 months. Disease recurrence was observed in eight of 28 patients (28·6%) with a mean time to recurrence of 7·28 months, and five of them died from the disease in a mean time of 13·2 months. After treatment, 14/28 patients could be analysed for HPV-16 E7-specific T-cell responses; of these, 10 received a combined chemotherapy and radiotherapy and four received only radiotherapy. The healthy group consisted of 16 women between 21 and 47 years (median age: 31 years) with reports of normal cytology.

Table 1. Characteristics of cervical cancer patients and healthy women (controls)

Group	Stage (FIGO)	Mean age (years)	Mean pretreatment tumour size (cm)
Pretreatment cervical cancer patients ($n = 32$)	IBI ($n = 3$)	54	1·5
	IIA ($n = 2$)	43	4·75
	IIB ($n = 9$)	46	4·6
	IIIB ($n = 18$)	44	7·24
Post-treatment cervical cancer patients ($n = 14$)	IBI ($n = 1$)	60	1·0
	IIB ($n = 5$)	41	4·75
	IIIB ($n = 8$)	41	9·00
Controls ($n = 16$)	Normal cytology	31	

Phenotype of monocyte-derived dendritic cells and HPV-16 E7 protein uptake

The flow cytometry analysis of monocytes showed a $CD14^+$ cell population with 90% purity (Fig. 1a). After 5 days of culture with IL-4 and GM-CSF, the obtained flow cytometry analysis of monocyte-derived iDCs was as follows: $CD1a^+$ population represented 85–90%; $CD86^+$ population comprised 20–70%; HLA-DR^+ 90–95%, and $CD83^{dim}$ 2–5%. The morphology of these cells was characterized by the presence of membrane projections and by size and complexity greater than of monocytes (Fig. 1a). The morphological and immunophenotypic characteristics of the iDCs of healthy women and cervical cancer patients showed no differences.

To confirm whether iDCs are able to take up the HPV-16 E7 protein, they were incubated with 10 μg/ml of the HPV-16 E7 protein and analysed at different periods of time for antigen capture by using indirect immunofluorescence. A high intensity of intracellular fluorescence was observed 2 hr after incubation with the antigen. No differences were observed in antigen presentation between 2 and 48 hr, because the fluorescence intensities were similar between these times (mean fluorescence intensity = 1680) (Fig. 1b). When the immunophenotype of these cells was examined after 48 hr, we observed a partial increase of HLA-DR and CD83 expression (data not shown). To obtain complete maturation of iDCs loaded with HPV-16 E7 protein, 2 hr after stimulation, 100 ng/ml LPS was added. After 48 hr, the FACS analysis showed that iDCs loaded with HPV-16 E7 protein significantly up-regulated expression of the surface markers HLA-DR, CD83 and CD86 (Fig. 1b). Furthermore, iDC activation was associated with HPV-16 E7 protein uptake 48 hr after the incubation.

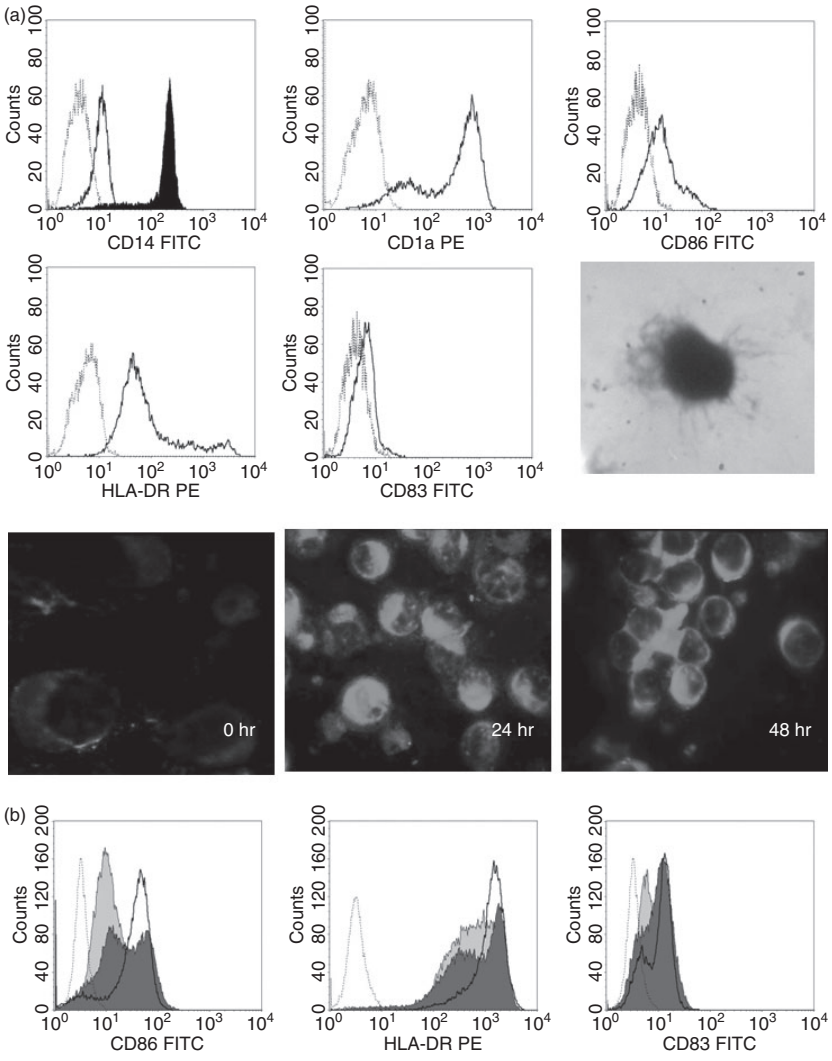

Figure 1. (a) Phenotype of monocyte-derived dendritic cells (DCs). The black histogram illustrates the expression of CD14 in monocytes at day 1, and the black line illustrates the expression of CD14 after 5 days. Level of expression of CD1a, CD86, HLA-DR and CD83 markers after 5 days. Isotype-matched control antibody is represented as a dotted line. Data are from a representative assay. Morphological characteristics of immature DCs (iDCs) at day 5 of differentiation. (b) Kinetics of human papillomavirus type 16 (HPV-16) E7 protein uptake and activation of mature DCs (mDCs). Indirect immunofluorescence of uptake kinetics of HPV-16 E7 protein by DCs during their maturation. The histograms illustrate the expression of CD86, HLA-DR, and CD83 markers after lipopolysaccharide (LPS) stimulus in loaded HPV-16 E7 DCs. Dotted line shows staining with isotype-matched control antibody, the grey and dark histograms show DCs after 0 hr and 24 hr treatment respectively and the dark line shows mDCs with the HPV-16 E7 protein at 48 hr. Data are from a representative assay.

No difference in frequencies of HPV-16 E7-specific Th1 cells between healthy women and cervical cancer patients

We used multiparametric flow cytometry analysis of intracellular cytokines to determine the frequencies of $CD4^+$ $CD69^+$ T cells producing IFN-γ (Th1 response) or IL-4 (Th2 response) in response to a short-term *in vitro* HPV-16 E7 stimulation of PBMCs from patients with cervical cancer and women with normal cytology (Fig. 2).

We observed positive responses to viral antigens in 3 of 16 (18·75%) healthy women. One of them showed specific Th1 responses when the PBMCs were stimulated with E7 protein-pulsed DCs and $E7_{79–98}$ peptide, the second one responded only to E7 protein and the last one responded only to $E7_{79–98}$ peptide. The responders mean frequencies to E7 protein and to $E7_{79–98}$ peptide were 0·045 ± 0·007% and 0·145 ± 0·015%, respectively. All control women were HPV DNA-negative; however, detectable levels of anti-HPV-16 L1 antibodies were presented only in the three Th1 responders, indicating a previous viral infection.

Before treatment, PBMCs from 31 of 32 cervical cancer patients were stimulated with the E7 peptides, from which 15 of 31 (48·4%) responded to any E7 peptide. Twelve of them (38·7%) showed a specific Th1 response to $E7_{79–98}$ peptide (responders mean frequency: 0·198 ± 0·048%), while five responded to $E7_{65–84}$ peptide (responders mean frequency: 0·050 ± 0·013%). A specific HPV-16 E7 protein Th1 response was found in 10 of 26 (38·4%) patients (responders mean frequency: 0·063 ± 0·016%) (Table 2), two of them responded also to $E7_{51–70}$ and $E7_{65–84}$ peptides, five of them responded also to $E7_{65–84}$ peptide and the other three responded also to $E7_{51–70}$ peptide at the same time.

When we compared the mean frequencies of HPV-16 E7-specific $CD4^+$ $CD69^+$ IFN-γ^+ T cells between patient and control groups, higher frequencies were observed in the patient group for all viral antigens except $E7_{51–70}$ peptide, but this difference was not statistically significant

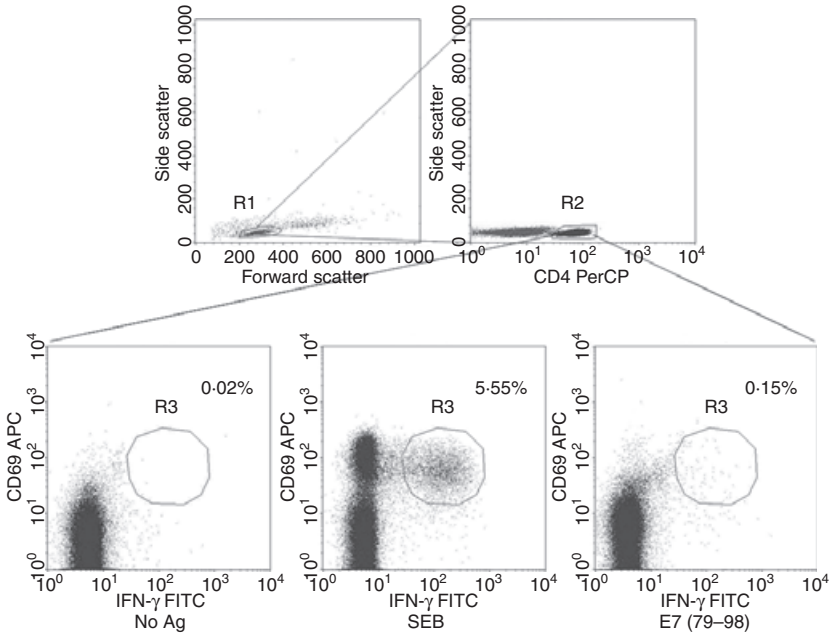

Figure 2. Multiparametric flow cytometric analysis; 30 000–50 000 gated $CD4^+$ events (R2) were acquired and analysed using CELL QUEST PRO software. The data are representative of pretreatment patients. Analysis of the percentage of human papillomavirus (HPV)-specific $CD4^+$ T cells was performed at the R3 gate.

Table 2. Frequencies of human papillomavirus type 16 (HPV-16) E7-specific T helper lymphocytes expressing interferon-γ (IFN-γ) or interleukin-4 (IL-4) in women with invasive cervical cancer before treatment and in healthy women (controls)

		Th1 (IFN-γ^+)		Th2 (IL-4^+)	
Group	Antigen	Responders (*n*)	Mean frequency ± SEM	Responders (*n*)	Mean frequency ± SEM
Controls	E7 protein	2	0·045 ± 0·007%	0	
(*n* = 16)	$E7_{51–70}$	0		0	
	$E7_{65–84}$	2	0·065 ± 0·005%	0	
	$E7_{79–98}$	2	0·145 ± 0·015%	0	
Cervical cancer	E7 protein	10^1	0·063 ± 0·016%	2	0·027 ± 0·003%
patients	$E7_{51–70}$	1^2	0·04%	0	
(*n* = 32)	$E7_{65–84}$	5^2	0·050 ± 0·013%	1	0·06%
	$E7_{79–98}$	12^2	0·198 ± 0·048%	5	0·150 ± 0·065%

Th1, T helper type 1; Th2, T helper type 2.
[1]Of 26 cervical cancer patients.
[2]Of 31 cervical cancer patients.

($P > 0{,}05$) (Fig. 3a). In addition, no differences were observed in the mean frequencies of $CD4^+$ $CD69^+$ IFN-γ^+ T lymphocytes between cervical cancer patients who were responders and who were non-responders, as well as in healthy women when SEB was used as antigen ($P > 0{\cdot}05$).

HPV-16 E7-specific Th2 responses were detected only in the cervical cancer patients group

Table 2 shows the mean frequencies of HPV-16 E7-specific $CD4^+$ $CD69^+$ $IL4^+$ T lymphocytes in the responders. In the healthy women group, Th2 response was not observed to either E7 protein or to peptides. In contrast, when the PBMCs from cervical cancer patients were stimulated with HPV-16 E7 protein, $E7_{65–84}$ peptide or $E7_{79–98}$ peptide, a Th2 response was detected in 2 of 26, 1 of 31 and 5 of 31 patients, respectively (Table 2). All Th2 responders also showed a Th1 responses.

Increase of HPV-16 E7-specific Th1 response in cervical cancer patients after treatment

Figure 4(a) shows the immune response to HPV-16 E7 in cervical cancer patients before and after treatment. When Th1 responses to $E7_{79–98}$ peptide were evaluated after treatment, a significantly higher number of patients were positives (10 of 14: 71·4%), in comparison with the response seen before treatment (4 of 14; 28·5%) ($P = 0{\cdot}039$) (Fig. 4b). Although a higher responders mean frequency of $CD4^+$ $CD69^+$ IFN-γ^+ T lymphocytes was observed after treatment in comparison with the one observed pretreatment (0·851 ± 0·515% versus 0·185 ± 0·098%, respectively) (Table 3), this difference

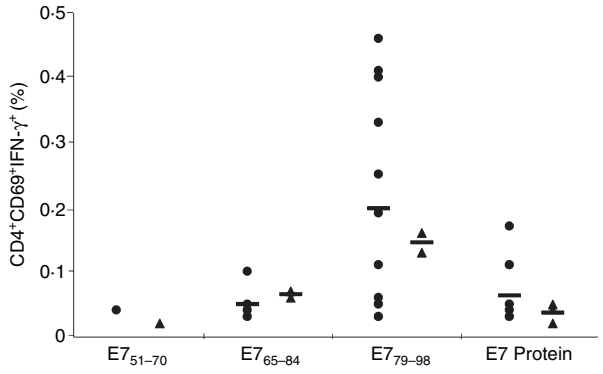

Figure 3. Frequencies of $CD4^+$ $CD69^+$ IFN-γ^+ E7-specific T cells in response to human papillomavirus type 16 (HPV-16) E7 antigens in cervical cancer patients (●) and the control group (▲). The solid lines represent the mean frequency. Frequencies of non-responders were below 0·02 and they are not shown in the graph.

was not statistically significant ($P > 0·05$). When HPV-16 E7 protein and $E7_{65–84}$ peptide were used as antigens, no differences were observed between pre- and post-treatment responses. After treatment, Th2 responses were observed in four cervical cancer patients (4 of 14; 28·5%), One of them was also positive before treatment (1 of 14; 7·1%) when $E7_{79–98}$ peptide was used as antigen ($P > 0·05$) (Fig. 4c). Interestingly, the mean frequencies before treatment were higher than those observed after treatment (0·225 ± 0·155% versus 0·178 ± 0·058%, respectively (Table 3), but this was not statistically significant ($P > 0·05$) (Table 3).

Analysis of survival and immune response to HPV-16 E7

We analysed the role of an HPV-16 $E7_{79–98}$-specific response on OS and long-term disease control in responders and non-responders. Univariate analysis of DFS and OS is shown in Table 4. Before treatment, a Th2 response to $E7_{79–98}$ peptide was significantly associated with DFS ($P = 0·027$) (Fig. 5). Although patients that showed Th1 responses to this peptide had a shorter survival in comparison with the non-responders, this difference was not significant. After treatment, decreased OS and DFS were observed between patients that showed Th1 or Th2 responses to $E7_{79–98}$ peptide, but these differences were not significant.

HLA class I reduced expression in cervical cancer patients before treatment

Finally, when the HLA I expression in cervical cancer patients was analysed, an absence of HLA I expression

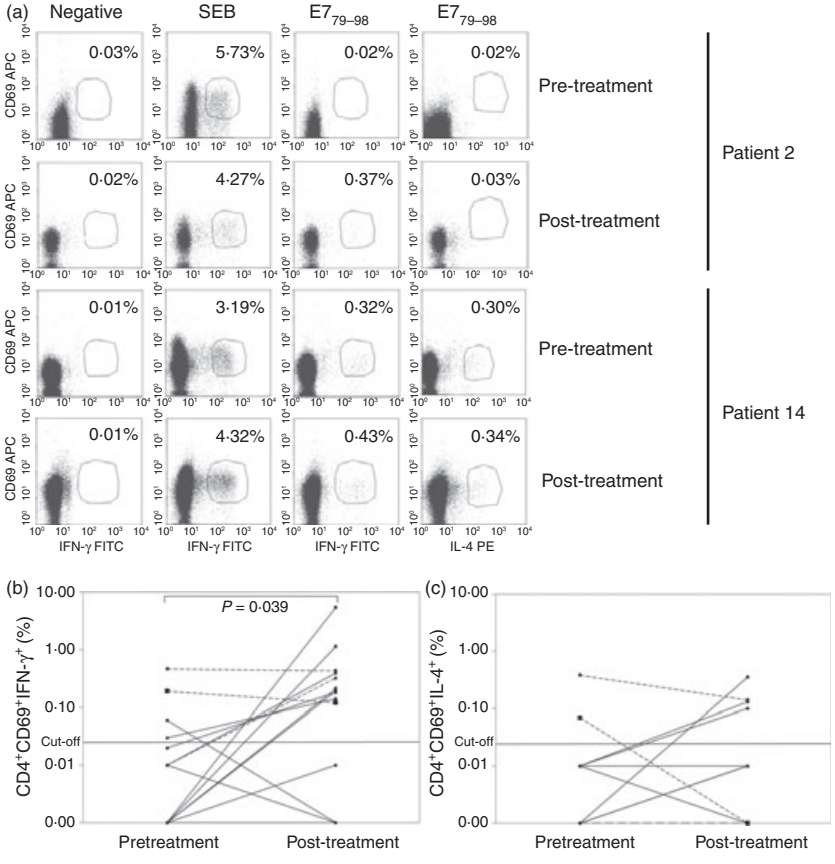

Figure 4. (a) Frequencies of $CD4^+$ $CD69^+$ IFN-γ^+ or $CD4^+$ $CD69^+$ IL-4 E7-specific T cells before and after treatment from two patients with cervical cancer. (b) Frequencies of $CD4^+$ $CD69^+$ IFN-γ^+ E7-specific T cells and (c) $CD4^+$ $CD69^+$ IL-4^+ E7-specific T cells in response to $E7_{79–98}$ peptide in cervical cancer patients before and after treatment. The dashed lines represent the patients that showed recurrence and the dashed line ended with a square symbol represents the patient who died.

Table 3. Frequencies of human papillomavirus type 16 (HPV-16) E7-specific T helper lymphocytes expressing interferon-γ (IFN-γ) or interleukin-4 (IL-4) in women with invasive cervical cancer before and after treatment

		Th1 (IFN-γ^+)		Th2 (IL-4^+)	
Group	Antigen	Responders (n)	Mean frequency ± SEM	Responders (n)	Mean frequency ± SEM
Pretreatment (n = 14)	E7 protein	5[1]	0·048 ± 0·016%	2[1]	0·03%
	$E7_{51–70}$	1	0·04%	0	
	$E7_{65–84}$	3	0·057 ± 0·022%	1	0·06%
	$E7_{79–98}$	4	0·185 ± 0·098%	1	0·255 ± 0·155%
Post-treatment (n = 14)	E7 protein	4[1]	0·043 ± 0·007%	0	
	$E7_{65–84}$	2	0·040 ± 0·010%	1	0·020%
	$E7_{79–98}$	10	0·851 ± 0·515%	4	0·178 ± 0·058%

[1]Of 12 cervical cancer patients.

Table 4. Overall survival (OS) and disease-free survival (DFS) according to human papillomavirus type 16 (HPV-16) E7-specific T helper response in women with invasive cervical cancer before and after treatment

	HPV-16 $E7_{79–98}$-specific response	n	40 months OS (%)	P*	n	40 months DFS (%)	P*
Pretreatment	Th1 response						
	Positive	10	70·0	0·157	10	60·0	0·288
	Negatives	18	88·9		18	77·8	
	Th2 response						
	Positives	5	60·0	0·126	5	40·0	0·027
	Negatives	23	87·0		23	78·3	
Post-treatment	Th1 response						
	Positives	10	81·8	0·536	10	70·0	0·303
	Negatives	4	100·0		4	100·0	
	Th2 response						
	Positives	5	80·0	0·763	5	60·0	0·446
	Negatives	9	77·8		9	77·8	

Th1, T helper type 1; Th2, T helper type 2.
*Log$P$*log rank test.

and a weak staining were observed in 11 of 18 (61·1%) and 4 of 18 (22·2%) patients, respectively; HLA expression was seen in only three samples from patients (16·7%) (Fig. 6).

Discussion

In this study, HPV-16 E7-specific T helper responses were detected in peripheral blood from patients with HPV-16-positive invasive cervical cancer and from healthy women. To analyse the pattern of type 1 (IFN-γ) and type 2 (IL-4) T-cell responses, we used a four-colour flow cytometric analysis for intracellular IFN-γ or IL-4 expression by $CD4^+$ $CD69^+$ T cells after *in vitro* stimulation with HPV-16 E7-loaded autologous DCs or directly with HPV-16 E7 synthetic peptides. Three synthetic peptides of 20 amino acids from the C-terminal region of HPV-16 E7 protein were employed: $E7_{51–70}$, $E7_{65–84}$, and $E7_{79–98}$. Between these peptides, $E7_{79–98}$ induced a major response in cervical cancer patients. These results were similar to those published by other authors, who defined the C-terminal region of the HPV-16 E7 protein as the more immunogenic region, with a high diagnostic and therapeutic potential.[12,23–26]

In accordance with previous data, E7-specific responses were detected in a low proportion of healthy subjects.[13] Three of 16 healthy women (18·75%) showed HPV-16 E7-specific $CD4^+$ $CD69^+$ IFN-γ^+ T cells. In these women, specific HPV-16 L1 IgG antibodies were detected, indicating previous infections with HPV-16. Although it is difficult to compare our results with those of previous works, because of methodological differences, we demonstrated, like de Boer *et al.* and Visser *et al.*[25,27], that a considerable number of HPV-16 DNA-positive cervical cancer patients present an E7-specific T helper cell response. In contrast, Jong *et al.* and Welters *et al.*[13,28] observed a low number of cervical cancer patients responding to E7 with T helper cell IFN-γ production. In our study, HPV-16 E7-specific $CD4^+$ IFN-γ^+ lymphocytes against one or more E7 peptides were detected in 15 of 31 (48·4%) patients with cervical cancer. In addition, no differences in response to superantigen (SEB) stimulation were observed between patients and healthy women, indicating a competent

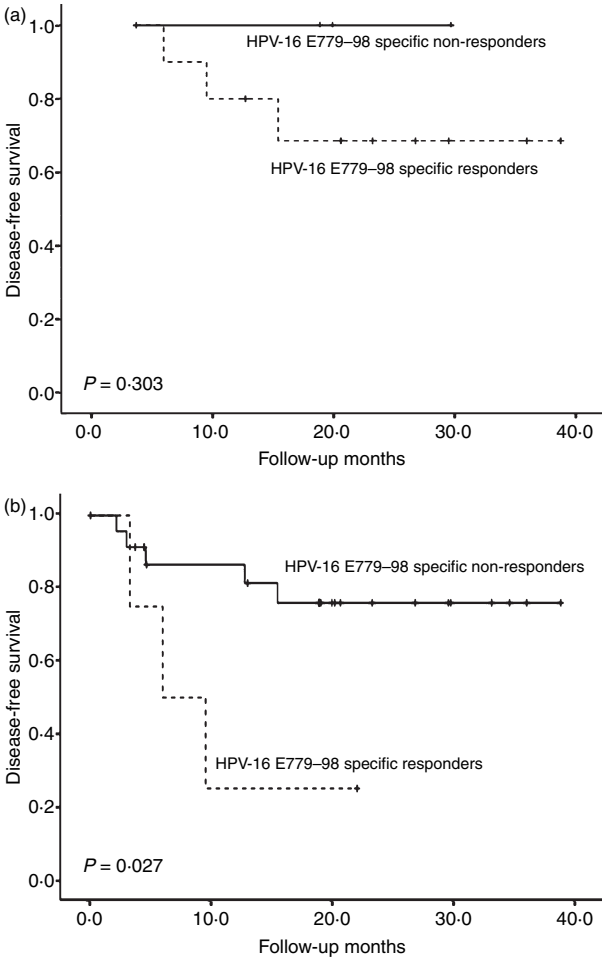

Figure 5. Disease-free survival with respect to human papillomavirus type 16 (HPV-16) $E7_{78–98}$-specific immune response. Kaplan–Meier curves of disease-free survival in months for responders and non-responders. (a) Th1 HPV-16 E7-specific immune response after treatment. (b) Th2 HPV-16 E7-specific immune response before treatment (*P*, log rank test).

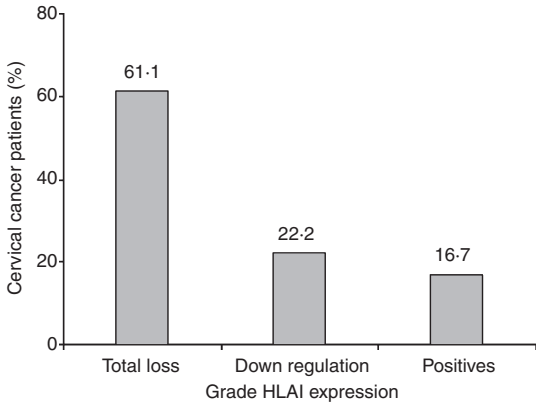

Figure 6. Analysis of human leucocyte antigen (HLA) I expression in cervical tumour cells. HLA I expression was analysed by immunohistochemistry in biopsies from patients with cervical cancer. Three expression levels were determined: total loss (negative), down-regulation (low expression) and positives.

immune system. However, the number of cervical cancer patients that responded to the HPV-16 E7 protein was low despite the fact that they were positive for HPV-16 DNA. These data support the hypothesis that probably the progression of cervical cancer associated with HPV is related to an alteration of the antigen-specific immune response at the tumour site. This immune response alteration can be explained by the existence of local immunosuppression, influenced by the presence of regulatory T cells, which secrete cytokines capable of impairing cellular immunity against HPV-16 E6/E7 oncoproteins.[29,30] Moreover, it is known that, during natural infection, HPV induces a negative regulation in the expression of major histocompatibility complex (MHC) class I molecules in the tumoral cells that might influence the immune response to HPV-16 E6/E7 oncoproteins.[31] We analysed the HLA I expression in cryopreserved biopsies from cervical cancer patients. Weak to absent HLA type I expression in cervical cancer patients was found in 83·3% of pretreatment analysed cases. We also confirm that the down-regulation of MHC I expression does not allow a good antigenic presentation, which could result in a poor antitumoral immune response.[31]

It has been reported that the progression to cervical cancer is associated with a shift from Th1 to Th2 cytokine production.[14,15] In our case, HPV-16 E7-specific $CD4^+$ IFN-γ^+ T-cell responses were detected in the control group as well as in cervical cancer patients; in contrast, an HPV-16 E7-specific $CD4^+$ IL-4^+ T-cell response was detected only in cervical cancer patients; however, this response was detected in a low number of patients (4 of 14). This result is probably the result of the low sensitivity of intracellular IL-4 detection by flow cytometry. Interestingly, in this study we observed that pretreatment patients who had an HPV-16 $E7_{79–98}$-specific Th2 response presented decreased disease-free survival. In a previous study we had observed a strong association between poor survival and the presence of antibodies IgG to $E7_{79–98}$ peptide.[2] Although our study is limited by the small number of patients, these results support the idea that a Th2-type cellular immune response could be useful as the predictor of recurrence and poor prognosis, clearly other studies will be needed to confirm our finding. Besides, to improve the sensitivity in this case it would be better to detect IL-13.

An increase of HPV-16 E7-specific Th1 cell responses after local invasive procedures in patients with premalignant cervical lesions and cervical cancer has been reported by Visser *et al.*[27] In our study, an immune-specific response against the HPV-16 $E7_{79–98}$ peptide was observed in 10 of 14 patients (71·4%) after treatment, and only four of 14 (28·5%) patients showed a specific

response before radiotherapy. This increase of the HPV-specific immune response could be the result of continuous stimulation of the immune system by a high antigenic load of the E7 oncoprotein and the MHC class I up-regulation caused by radiotherapy. These results support the Santin hypothesis in which the cellular response induced by ionizing radiation including the up-regulation of viral proteins as well as of MHC I surface antigens on cervical carcinoma cells could increase the immunogenicity of HPV-infected tumour cells and contribute to the efficacy of radiotherapy in the treatment of cervical carcinoma.[18]

It has been reported that the HPV-16-specific T-cell responses of patients with squamous intraepithelial lesions (SIL) before and after treatment did not change greatly in terms of peptide specificity, range of response observed, or number of responding patients, which suggests that treatment itself had no significant positive or negative effect on these responses. However, it was observed that the acquisition of an $E7_{70–98}$ peptide-specific T-cell response in a high number of patients with recurrent disease does not protect against reinfection by HPV or recurrence of cervical disease.[26] In our study, in spite of an increase of HPV-16 $E7_{79–98}$-specific immune response in a high number of patients after treatment, this response was not correlated with a successful clinical response. Besides, the higher mean frequency of HPV-16 E7-specific Th1 cells after treatment compared to pretreatment was not statistically different. It is possible that the number of Th1 precursors is not enough to eliminate the tumour cells. Our results agree with those from a study by Visser *et al.*[27] and support the idea that in cervical cancer patients the presence of a relatively strong cellular immune response against HPV-16 E7 might be a marker for the severity of disease, more than a reflection of the host's capability of controlling tumour growth.

In summary, our data demonstrate an association between radiotherapy, with or without concurrent cisplatin, and the increase of HPV-specific immune response in cervical cancer patients after treatment. This observation supports the hypothesis that lysis of tumour cells by gamma-radiation could lead to tumoral antigen release and reactivating the immune response;[17] however, this increase of specific immune response would not be enough for controlling completely the apparition of disease after treatment. Our results underline the importance of other immune factors that could influence the tumoral immune response, such as the down-regulation of HLA I in tumour cells and the presence of regulatory T cells in the tumoral microenvironment. These results could have important implications during the development of new therapeutic strategies such as immunotherapy because although there is a reactivation of the immune response after treatment, this response is not enough to control tumour recurrence.

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