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Frequencies and role of regulatory T cells in patients with (pre)malignant cervical neoplasia

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Summary

Oncogenic human papillomavirus (HPV)-infection is crucial for developing cervical cancer and its precursor lesions [cervical intraepithelial neoplasia (CIN)]. Regulatory T cells (T_{regs}) might be involved in the failure of the immune system to control the development of HPV-induced cancer. We investigated frequencies, phenotype and activity of T_{regs} in patients with cervical neoplasia. CIN and cervical cancer patients showed increased $CD4^+/CD25^{high}$ T cell frequencies in peripheral blood and $CD4^+$ T cell fraction. These $CD4^+/CD25^{high}$ T cells represent T_{regs} as demonstrated by their low proliferation rate, low interferon (IFN)-γ/interleukin (IL)-10 ratio, high expression of CD45RO, GITR, CTLA-4, forkhead box P3 (FoxP3) and low CD45RA expression. Moreover, in $HPV16^+$ cervical cancer patients, *in-vitro* depletion of $CD25^+$ T cells resulted in increased IFN-γ T cell responses against HPV16 E6- and E7 peptides. Thus, increased frequencies of T_{regs} in cervical cancer patients may indeed suppress HPV-specific immunity. Longitudinal analysis of $CD4^+/CD25^{high}$ T cell frequencies in patients showed a modest decline 1 year after curative surgery or chemoradiation. This study demonstrates increased frequencies and suppressive activity of T_{regs} in cervical cancer. These results imply that T_{regs} may suppress the immune control of cervical neoplasia and furthermore that suppression of immunity by T_{regs} will be another hurdle to overcome in therapeutic immunization strategies against cervical neoplasia.

Keywords: $CD4^+$ T cell, $CD8^+$ T cell, cervical cancer, HPV, regulatory T cells

Introduction

Natural regulatory T cells (T_{regs}), characterized by co-expression of CD4 and CD25, play an important role in immune homeostasis [1–4]. In animal tumour models, elevated frequencies of T_{regs} have been demonstrated and T_{reg} depletion increased the anti-tumour immune responses [5,6]. These observations led to the hypothesis that cancer patients have an enlarged population of T_{regs} inhibiting tumour-specific T cell responses [1–3]. A recent study showed that $CD4^+/CD25^+$ T_{regs} control the induction of antigen-specific T-helper responses in cancer patients [7]. Intratumoral T_{regs} have been demonstrated in ovarian cancer patients [8] and an enlarged population of T_{regs} in peripheral blood of patients with different types of cancer [9–13].

Infection with oncogenic human papillomavirus (HPV) is involved in cervical carcinogenesis; HPV DNA can be detected in $\geq$ 99% of all cervical cancers [14,15]. Most women infected with oncogenic HPV types clear the infection and do not develop (pre)malignant cervical neoplasia. The importance of the immune system in HPV clearance is demonstrated by observations that immunocompromised women fail more often to clear HPV infections and have an increased risk of developing cervical cancer [16].

The E6 and E7 oncoproteins of HPV play a crucial role in the transformation and maintenance of the malignant phenotype [15]. Several reports showed impaired cellular immunity against the HPV16 E6 and/or E7 oncoproteins in cervical cancer patients [17–20]. It has been suggested that impaired cellular immunity against these oncoproteins is responsible for the failure to eradicate HPV infections,

Table 1. Patient characteristics.

CIN patients					Cervical cancer patients				
Patient no.	Age (years)	Diagnosis	Grade	HPV16 status	Patient no.	Age (years)	Diagnosis	Stage	HPV16 status
1	26	CIN	I	neg	1	37	SCC	IA1	pos
2	28	CIN	I	neg	2	30	SCC	IA1	pos
3	27	CIN	I	neg	3	71	SCC	IB1	pos
4	50	CIN	I	?	4	36	SCC	IB1	pos
5	45	CIN	I	?	5	33	SCC	IB	neg
6	27	CIN	I	neg	6	40	SCC	IB1	neg
7	41	CIN	I	pos	7	75	SCC	IB1	pos
8	48	CIN	I	?	8	60	SCC	IB1	pos
9	34	CIN	II	pos	9	35	SCC	IB1	pos
10	34	CIN	III	neg	10	45	SCC	IB1	neg
11	33	CIN	III	pos	11	36	SCC	IB1	neg
12	45	CIN	III	pos	12	33	SCC	IB2	pos
13	40	CIN	III	?	13	26	SCC	IBII	pos
14	38	CIN	III	neg	14	54	SCC	IIA	pos
15	40	CIN	III	pos	15	43	SCC	IIB	pos
16	40	CIN	III	pos	16	70	SCC	IIB	neg
17	35	CIN	III	?	17	43	SCC	IIB	pos
18	30	CIN	III	?	18	46	SCC	IIB	pos
					19	36	SCC	IIB	pos
					20	48	SCC	IIB	pos
					21	51	SCC	IIB	pos
					22	41	SCC	IIB	pos
					23	45	SCC	IIB	neg
		Avg 35 ± 8					Avg 45 ± 13		

CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma; HPV, human papillomavirus.

leading subsequently to (pre)malignant cervical neoplasia [18]. In other studies, significant cellular immune responses against HPV16 E6 and/or E7 were demonstrated in cervical intraepithelial neoplasia (CIN) and cervical cancer patients [21–23]. However, these responses seem unable to clear HPV infections [21–23]. Interestingly, we observed that (suboptimal) T cell responses against HPV in CIN and cervical cancer patients can be enhanced by invasive surgical procedures [21].

$CD4^+/CD25^+$ T_{regs} have been found in tumour-draining lymph nodes of cervical cancer patients [24,25]. Immunohistochemistry also revealed the presence of $CD25^+$ T_{regs} in infiltrate associated with CIN lesions [26]. However, in these studies frequencies and phenotypes of T_{regs} in CIN or cervical cancer patients were not compared to healthy controls; nor was suppression of (HPV-specific) cellular immune responses by T_{regs} determined.

To elucidate the role of T_{regs} in developing (pre)malignant cervical neoplasia, we evaluated frequencies, phenotype and suppressive activity of $CD4^+/CD25^+$ T cells in peripheral blood of patients with cervical cancer or CIN and healthy controls. Longitudinal analysis of T_{reg} frequencies was performed during and following therapy. We also investigated if the previously observed suboptimal T cell responses against HPV can be ascribed to increased frequencies/activities of T_{regs}.

Materials and methods

Ethical approval

The study was approved by the local medical ethical committee of the University Medical Center Groningen (UMCG). Written informed consent was obtained from all patients.

Patients

CIN and cervical cancer patients (Table 1) were recruited from the out-patient clinic of the gynecology department at the UMCG, as described previously [21]. In the Netherlands, cervical smears are classified according a modified Papanicolaou system in which borderline dyskaryosis corresponds well with the Bethesda classification of atypical squamous cells of undetermined significance, mild dyskaryosis with low-grade squamous intraepithelial lesions and moderate and severe dyskaryosis and carcinoma *in situ* with high-grade squamous intraepithelial lesions [27]. Patients referred with cervical carcinoma were staged according FigO criteria [28]. In general, patients with FigO stages Ib/IIa were treated by radical surgery and patients with stages IIb–IV were treated with chemoradiation. Radiotherapy consisted of 50 Gy in 25 fractions, five fractions a week, combined with

two fractions of brachytherapy if indicated. In addition to radiotherapy, patients received 40 mg/m^2 of cisplatin per week for 6 weeks.

Female healthy volunteers (age 39 ± 11 years) were recruited from the departments of gynecology and medical microbiology of the UMCG.

Isolation of cell subsets

Heparinized blood (50 ml) was obtained and peripheral blood mononuclear cells (PBMC) were isolated with a Ficoll-density gradient. PBMC were cryopreserved using standardized conditions enabling batchwise analysis at a later time.

Using fluorescent activated cell-sorting, thawed PBMC of healthy controls and patients with (pre)malignant cervical neoplasia were separated into CD4$^+$/CD25^{neg} T cells, CD4$^+$/CD25^{low} T cells and CD4$^+$/CD25^{high} T cells. PBMC were stained with αCD4-APC (IQ Products, Groningen, the Netherlands) and αCD25-fluorescein isothiocyanate (FITC) antibodies (BD Biosciences, San Diego, CA, USA). Cells of interest were isolated with a Dako-Cytomation MoFlo High-Speed Sorter (Glostrup, Denmark), using gate-settings as described previously [13,29].

Flow cytometry

PBMC were stained with αCD25-FITC (BD Biosciences), αCD152-PE (CTLA4; BD Biosciences), anti-glucocorticoid-induced tumour necrosis factor (TNF) receptor family-related gene (GITR)-phycoerythrin (PE) (R&D Systems, Minneapolis, OK, USA), αCD45RO-PE (IQ Products), αCD4-antigen-presenting cell (APC) (IQ Products), anti-forkhead box P3 (FoxP3)-PE (eBioscience) and isotype controls to determine the immunophenotype of the different CD25 T cell subsets. Flow cytometry was performed and cells were measured with a fluorescence activated cell sorter (FACS)Calibur (BD Biosciences). Cells were analysed using CellQuest software (BD Biosciences).

Cell cultures, cytokine- and proliferation assays

Isolated CD4$^+$/CD25 T cell subsets were cultured at a density of $2{\cdot}5 \times 10^4$ cells/well in 96-well round-bottomed plates (Nunc, Rochester, NY, USA). Cells were cultured in a volume of 200 µl RPMI-1640 (Gibco, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS) (BioWhittaker, Verviers, Belgium), penicillin/streptomycin and 50 µM β-mercaptoethanol.

Cells were stimulated with 0·75 µg/ml αCD3/1 µg/ml αCD28 (Sanquin Research, Amsterdam, the Netherlands). Culture supernatants were harvested after 3 days and cell proliferation was measured by overnight [^{3}H]-thymidine incorporation (1 µCi/well; Amersham, Bucks, UK). Labelled cells were harvested and [^{3}H]-thymidine incorporation measured with a liquid scintillation counter (Canberra-Packard, Meriden, CT, USA).

Cytokines were measured in culture supernatants using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Sanquin Research).

Expansion and detection of interferon (IFN)-γ-producing HPV16E6- and E7-specific T cells

For expansion and detection of HPV16 E6- and E7-specific T cells, we adapted an assay developed previously for detection of Epstein–Barr virus-specific CD4$^+$ and CD8$^+$ T cells [30,31]. HPV16 E6- or E7-specific T cells were stimulated using 15-mer peptides with an 11-aa overlap spanning the complete sequence of HPV16 E6 (37 peptides) or E7 (22 peptides) protein. Peptides were synthesized by Mimotopes/Perbio Sciences. Purity (> 90%) and sequences were verified by high performance liquid chromatography (HPLC)/mass-spectrometry. Peptides were dissolved in dimethylsulphoxide (DMSO) and pooled (final concentration of 1 mg/ml of each peptide). Stimulations with peptide pools and medium were performed in the presence of co-stimulation (2 µg/ml αCD28). As a negative control, cells were stimulated with medium and co-stimulation only. As a positive control, cells were stimulated with 10 ng/ml phorbol myristate acetate (PMA)/2 µg/ml ionomycin.

To expand HPV16 E6- or E7-specific T cells, total PBMC or CD25$^+$ cell-depleted PBMC were cultured for 12 days in the presence of E6 or E7 peptide pools. Culture medium consisted of RPMI-1640 (Gibco) supplemented with penicillin/streptomycin, 50 µM β-mercaptoethanol and 10% human pool serum (Sigma, Zwijndrecht, the Netherlands; complete medium). Cells were cultured at 2×10^5 PBMC/well in 100 µl complete medium in 96-well round-bottomed plates at 37°C and 5% CO_2. The peptide pool (at 2 µg/ml of each peptide) was added on days 0 and 6. Interleukin (IL)-2 (10 U/ml) was added on days 3, 6 and 9. On day 12, cells were pooled, washed in RPMI-1640 and incubated overnight in complete medium.

On day 13, the number of IFN-producing cells were determined by intracellular cytokine staining; 10^6 cells were stimulated in 500 µl complete medium for 6 h with HPV16 E6 or E7 peptide pools (at 2 µg/ml of each peptide) and αCD28 (2 µg/ml; Sanquin Research) as co-stimulation. After 1 h, brefeldin-A (Golgiplug; BD Biosciences) was added at a dilution of 1 : 1000 to allow cytokine accumulation in the cytosol. After stimulation, cells were washed in phosphate-buffered saline (PBS) + 0·5% bovine serum albumin (BSA), permeabilized (FACS permeabilizing solution; BD Biosciences), washed again and stained with αCD3-PE/Cy5 (Sanquin Research), αCD4-APC (IQ Products), αCD8-FITC (Sanquin Research) and anti-IFN-PE (IQ Products). Cells were washed again, fixed (Cellfix; BD Biosciences) and 2×10^5 events were acquired on a FACSCalibur and data analysed using CellQuest software.

Enzyme-linked immunospot (ELISPOT) to determine HPV16 E6- and E7-specific T cell responses

Cryopreserved PBMC were thawed and $CD25^+$ T cells were depleted using anti-CD25 Microbeads (Miltenyi-Biotec, Bergisch-Gladbach, Germany) according the manufacturer's instructions. This procedure leads to complete removal of $CD4^+/CD25^{high}$ T cell fractions. Unseparated PBMC and CD25-depleted were seeded at a density of $1{\cdot}5 \times 10^6$ cells/well in a 24-well plate (Corning Life-Sciences, Schiphol-Rijk, the Netherlands) in 1·5 ml complete medium in the presence or absence of HPV16 E6 or E7 peptide-pools (10 μg/ml of each peptide).

After 4 days of incubation, PBMC were harvested, washed and seeded in quadruplicate at a density of 1×10^5 cells/well in a coated ELISPOT-plate (Nunc, Silent-screen, Rochester, NY, USA) with an IFN-γ catching antibody (IFN-γ ELISPOT assay; Sanquin Research). The assay was performed further according the manufacturer's instructions (Sanquin Research). ELISPOT plates were analysed with a fully automated imaging device (A.EL.VIS, Hanover, Germany). The background in medium control wells was below 10 spots/1×10^5 cells. Specific spots were calculated using criteria similar to other studies [17–19,21]. Specific responses were calculated by subtracting the mean number of spots ± 2 s.d. of medium control wells from the mean number of spots of experimental wells. Response against HPV16 E6 or E7 peptide-pools were considered positive when the number of specific spots was $\geq 10/1 \times 10^5$ cells and the response in the experimental wells was at least two times the background levels. As a positive control, PBMC were stimulated with a memory recall mix (MRM; kind gift of Dr S. van der Burg, LUMC, the Netherlands), consisting of a mixture of *Tetanus toxoid* (0·75 LF/ml), *Mycobacterium tuberculosis* sonicate (2·5 μg/ml) and *Candida albicans* (0·005%).

Establishing HPV16 status

HPV16 status was established by HPV16-specific polymerase chain reaction (PCR) on DNA isolated from cervical scrapings obtained at the patient's initial visit. The scraped cells were suspended in 5 ml PBS and kept on ice. The HPV16-specific PCR was performed as described previously [21]. HPV16-primers [product 152 base pairs (bp)] were: sense: TGCTAGTGCTTATGCAGCAGCAA, anti-sense: ATTTACTGCAACATTGGTAC.

Statistical analysis

Differences between groups were determined using the Mann-Whitney *U*-test and χ^2 test. Significance was determined as $P < 0{\cdot}05$.

Results

Frequencies of $CD4^+/CD25^+$ T cells in peripheral blood of healthy controls, patients with CIN and cervical cancer

$CD4^+/CD25^+$ and $CD4^+/CD25^{high}$ T cells (Fig. 1a,d) were examined in patients with CIN, cervical cancer and healthy controls, using gate-settings as described previously [13,29]. As shown in Fig. 1, $CD4^+/CD25^+$ (Fig. 1b,c) and $CD4^+/CD25^{high}$ T cells (Fig. 1e,f) were significantly increased in both total PBMC (Fig. 1b,e) and $CD4^+$ T cell fraction (Fig. 1c,f) of patients with CIN and cervical cancer compared to healthy controls.

Immune phenotype and FoxP3 expression of $CD4^+/CD25$ T cell subsets

$CD4^+/CD25^{high}$ T cells of representative CIN patients, cervical cancer patients and healthy controls were characterized further for the expression of CD45RO, GITR, CTLA-4 and CD45RA. The three first molecules are expressed relatively highly and CD45RA is expressed relatively low on T_{regs} [1–3,9–13,29]. $CD4^+/CD25^{high}$ T cells of patients and controls expressed increased levels of CD45RO, CTLA-4 and GITR compared to $CD4^+/CD25^{neg}$ T cells (data not shown). Also, as the $CD4^+/CD25^{high}$ T cells expressed much lower CD45RA levels than $CD4^+/CD25^{neg}$ T cells (data not shown), the $CD4^+/CD25^{high}$ T cells of CIN and cervical cancer patients phenotypically resemble T_{regs}.

The transcription factor FoxP3 is considered to be a specific marker for T_{regs} [1,32,33]. Therefore, we analysed FoxP3 expression in the $CD4^+/CD25$ T cell subsets (Fig. 2, upper panel). In both healthy controls and cervical cancer patients, the majority of $CD4^+/CD25^{high}$ T cells expressed FoxP3, while $CD4^+/CD25^{neg}$ T cells and $CD4^+/CD25^{low}$ T cells expressed FoxP3 at very low and moderate levels, respectively (Fig. 2, lower panel).

Cytokine profiles of $CD4^+/CD25^{neg}$ and $CD4^+/CD25^{high}$ T cell subsets

In general, T_{regs} are characterized by low proliferation, low IFN-γ production and robust IL-10 production [1–3,9–13,29]. $CD4/CD25^{neg}$ and $CD4^+/CD25^{high}$ T cells were isolated by cell-sorting (Fig. 3a) to study their functionality *in vitro*.

The $CD4^+/CD25^{high}$ subset showed a low proliferation rate (Fig. 3b), high levels of IL-10 production (Fig. 3c) and low IFN-γ production (Fig. 3d). The $CD4^+/CD25^{neg}$ subset, on the other hand, showed the opposite profile. Although $CD4^+/CD25^{high}$ and $CD4^+/CD25^{neg}$ cells of cervical cancer patients, on average, produced lower cytokine levels, the cytokine profile of these T cell subsets was similar compared to these subsets in healthy controls and patients with CIN.

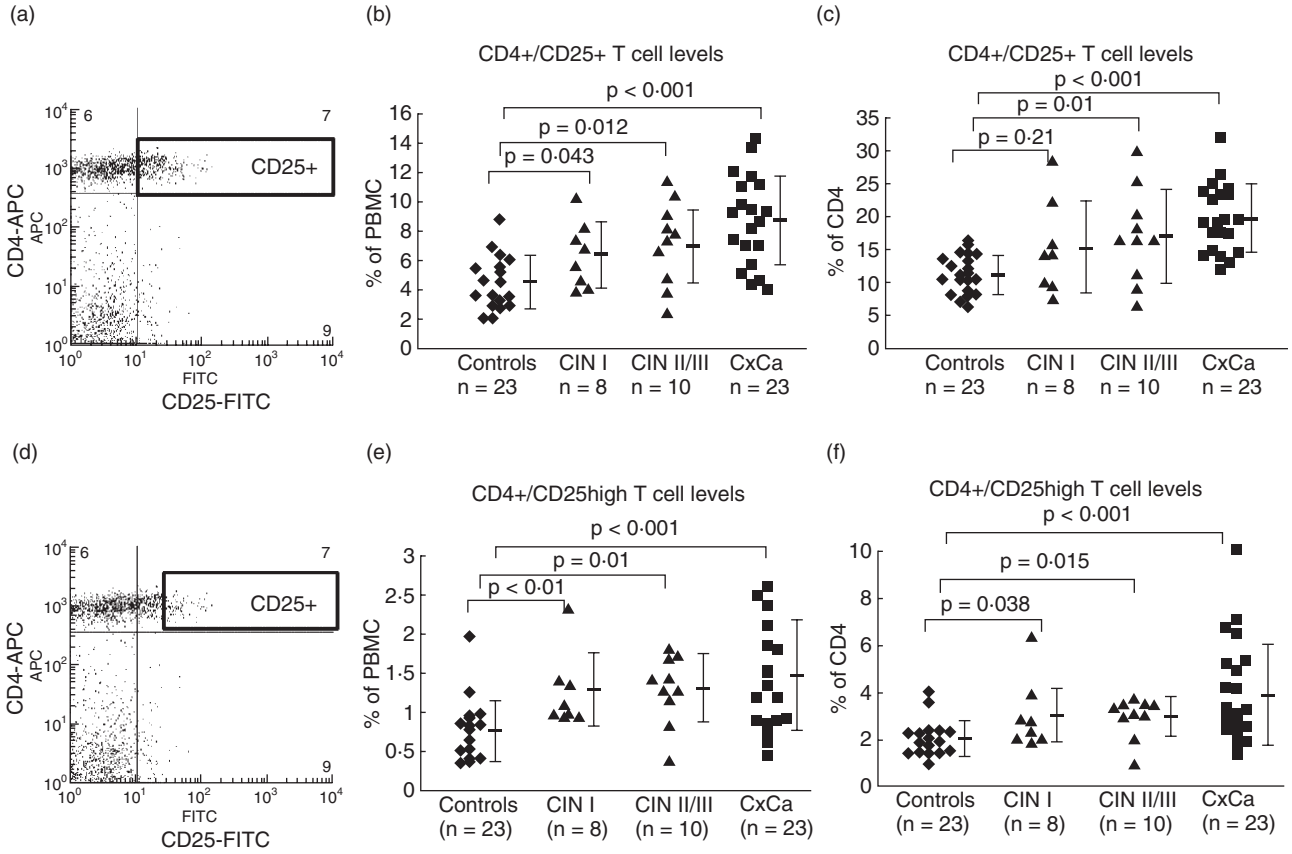

Fig. 1. (a, d) Gate-settings for calculating $CD4^+/CD25^+$ and $CD4^+/CD25^{high}$ T cell frequencies. Markers to establish $CD4^+/CD25^+$ T cell frequencies are set on the isotype control. (b, c) $CD4^+/CD25^+$ T cell frequencies in total peripheral blood mononuclear cells (PBMC) (b) and $CD4^+$ T cell population (c). (e, f) $CD4^+/CD25^{high}$ T cell frequencies in total PBMC and $CD4^+$ T cell population, respectively. Analysis was performed with PBMC collected before invasive therapy. Differences between groups were analysed using the Mann–Whitney *U*-test.

Effect of $CD25^+$ T cell depletion on HPV16 E6/E7-specific and general T cell responses

Using IFN-γ ELISPOT analysis, we determined the influence of $CD4^+/CD25^+$ T cells on HPV16 E6- and E7-specific T cell responses in patients with cervical cancer.

In vitro, depletion of $CD25^+$ T cells enhanced IFN-γ T cell responses against HPV16 E6 and/or E7 peptide-pools in 50% (five of 10) of the HPV16-DNA^+ cervical cancer patients (Table 2; $P = 0{\cdot}05$ compared to $HPV16^{neg}$ cervical cancer patients and $P = 0{\cdot}07$ compared to the controls). In $HPV16^{neg}$ cervical cancer patients no significant responses against HPV16 E6 and E7 peptide-pools were observed, and in only one healthy control $CD25^+$ T cell depletion enhanced the HPV16 E6-specific T cell response (Table 2).

In a number of HPV16-DNA^+, HPV16-DNA^{neg} and control donors, depletion of $CD25^+$ T cells enhanced MRM-specific T cell responses (Table 2). In one patient (patient 7) and three controls (controls 3, 5, 10) depletion of $CD25^+$ T cells caused a decrease of MRM-specific T cell responses. This is probably due to depletion of $CD25^+$ activated memory T cells.

Using standard IFN-γ ELISPOT analysis the total T cell response is measured, not discriminating between $CD4^+$ and $CD8^+$ T cell responses. As T_{regs} control both $CD4^+$ and $CD8^+$ T cell responses [1–4], we determined if depletion of $CD25^+$ T cells enhances HPV-specific T cell responses of both cell subsets.

All cervical cancer patients thus analysed displayed low, yet detectable $CD4^+$ T cell responses against HPV16 E6 and/or E7 peptide-pools (Table 3). As observed by others [19–22], we also found higher $CD4^+$ and $CD8^+$ T cell responses against the E6 peptide pool compared to the E7 peptide pool (Table 3). Upon depletion of $CD25^+$ cells, the response was enhanced in the $CD4^+$ T cell fraction in three of four patients. Patients 2, 4 and 20 showed CD8 responses against the HPV16 E6 and/or E7 peptide pools. However, in only patient 2 was the E6-specific response enhanced after $CD25^+$ cell depletion. Background IFN-γ production was not enhanced significantly by $CD25^+$ cell depletion. Although the results for the HPV16 E6- and E7-specific $CD8^+$ responses are inconclusive, these results suggest that $CD25^+$ T cells can at least suppress the HPV16 E6- and/or E7-specific $CD4^+$ T cell responses.

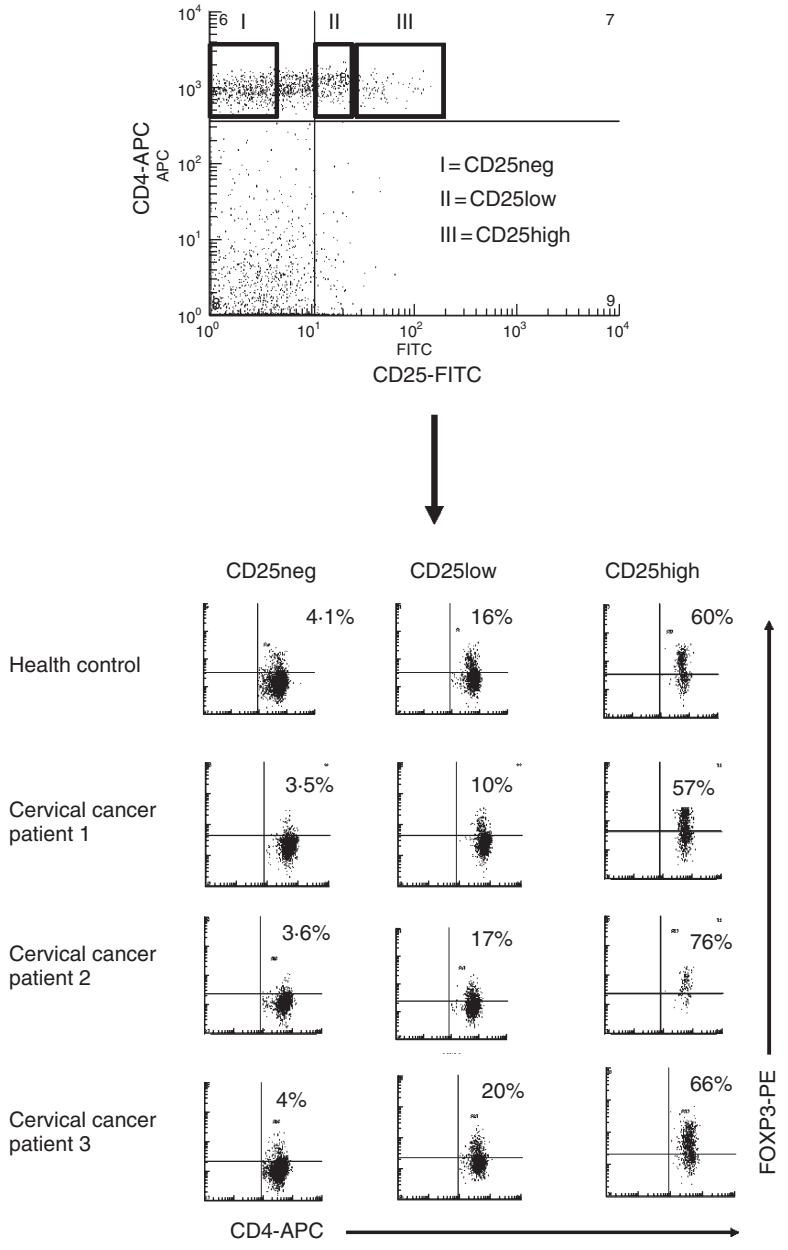

Fig. 2. Forkhead box P3 (FoxP3) expression was measured by flowcytometry in CD4$^+$/CD25 subsets of a healthy control and three cervical cancer patients with peripheral blood mononuclear cells collected before invasive therapy. The upper panel shows gate-settings for CD4$^+$/CD25^{neg}, CD4+/CD25^{low} and CD4+/CD25^{high} cells. Within the T cell fractions FoxP3 expression was analysed (lower panel).

Longitudinal follow-up of CD4$^+$CD25^{high} T cell frequencies in patients with cervical cancer

Longitudinal analyses of CD4$^+$/CD25^{high} T cell frequencies were performed in four healthy controls and 10 cervical cancer patients during and following therapy. In healthy controls the CD4$^+$/CD25^{high} T cell population appeared very stable (Fig. 4a). In two patients with cervical cancer who underwent curative surgery only, with no sign of recurrence for more than 2 years, CD4$^+$/CD25^{high} T cell frequencies showed a modest decline following curative surgery (Fig. 4b,c). CD4$^+$/CD25^{high} T cell frequencies showed strong fluctuation in patients treated with chemoradiation (RCT; Fig. 4d–k). In six patients the CD4$^+$/CD25^{high} T cell frequencies increased after radiochemotherapy (Figs 4d–h, k); in two patients these frequencies remained stable (Fig. 4i,j). After completion of chemoradiation, CD4$^+$/CD25^{high} T cell frequencies did not fall below the levels as measured before chemoradiation in all eight patients.

Discussion

In this study we provide evidence that patients with CIN and cervical cancer have increased CD4$^+$/CD25^{high} T_{reg} frequencies in their peripheral blood compared to healthy controls. Our data show that these CD4$^+$/CD25^{high} T cells, but not CD4$^+$/CD25^{low} T cells, of CIN and cervical cancer patients display all T_{reg} characteristics. The CD4$^+$/CD25^{high} T cells

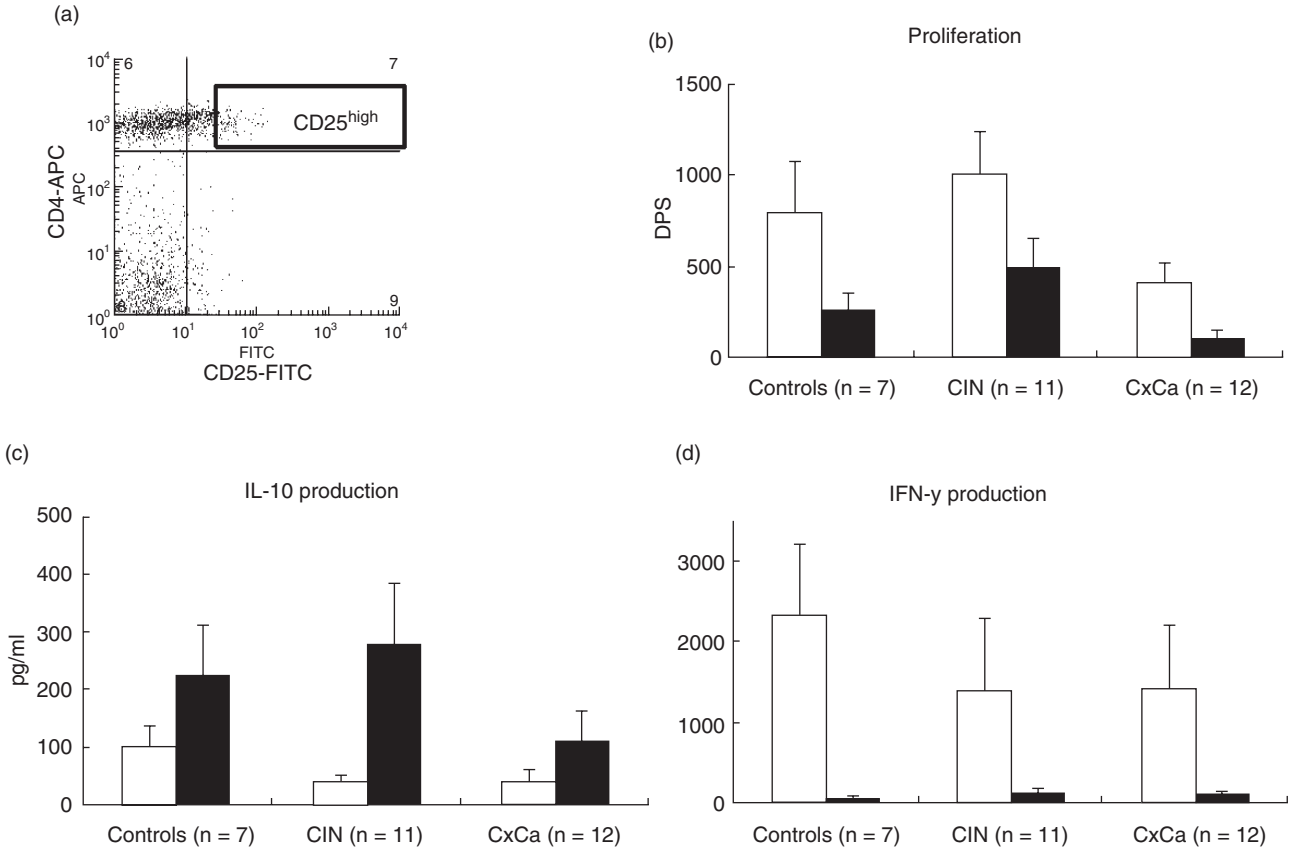

Fig. 3. (a) Gate-settings used for sorting the different $CD4^+/CD25$ T cell populations. White bars represent the $CD25^{neg}$ T cells and the black bars the $CD25^{high}$ T cells. Cell-sorting experiments were performed with peripheral blood mononuclear cells (PBMC) collected before invasive therapy of seven representative controls, 11 representative patients with cervical intraepithelial neoplasia and 12 representative patients with cervical cancer. Proliferation (b) is expressed as dissociations per second (dps; mean ± s.e.m.), interleukin-10 (c) and interferon-γ (d) production as pg/ml (mean ± s.e.m.).

express high levels of CD45RO, GITR and CTLA-4. Furthermore, these $CD4^+/CD25^{high}$ T cells produce high levels of IL-10, low levels of IFN-γ and have a low rate of proliferation. Moreover, this subset is highly positive for the T_{reg}-specific marker FoxP3.

It has been shown that *in vitro* $CD25^+$ T cell depletion enhanced virus-specific $CD4^+$ and/or $CD8^+$ T cell responses [33]. Moreover, in melanoma patients, $CD4^+/CD25^+$ T_{regs} control tumour-specific $CD4^+$ T cell responses [7]. We therefore hypothesized that increased T_{reg} numbers in cervical cancer patients might be responsible for impaired cellular immunity against HPV. Indeed, *in vitro,* depletion of $CD25^+$ T cells, enhanced HPV16 E6- and/or E7-specific T cell responses in 50% of the $HPV16\text{-}DNA^{pos}$ cervical cancer patients. This enhancement did not occur in $HPV16\text{-}DNA^{neg}$ cervical cancer patients, while only one healthy control showed an enhanced E6-specific T cell response, probably reflecting T cell memory of previous HPV-infection(s). Our results indicate that suppression of HPV16 E6/E7-specific T cell responses by $CD25^+$ cells was at the level of $CD4^+$ and possibly also $CD8^+$ T cells. The increased IFN-γ responses could not be explained by enrichment of responder T cells after $CD25^+$ cell depletion, because relative changes of $CD4^+$ T and $CD8^+$ T cells remained below 4% and 2%, respectively (data not shown).

$CD25^+$ T cell depletion also enhanced T cell responses against MRM in a considerable number of cervical cancer patients and healthy controls. In one patient (patient 7) and three controls (controls 3, 5 and 10), $CD25^+$ T cell depletion resulted in a decrease of the MRM-specific T cell response. In view of the fact that activated memory T cells express increased levels of CD25, this decrease might be due to depletion of activated memory T cells.

The simultaneous increase of HPV16 and MRM-specific T cell responses in $HPV16^+$ cervical cancer patients after $CD25^+$ T cell depletion indicate that suppression of cell-mediated immunity by T_{regs} in these patients is not antigen-specific. However, our results imply that increased frequencies of T_{regs} in cervical cancer patients might suppress the immunological control of cervical neoplasia. Insufficient cell numbers were available to characterize further the suppression of general immune responses by T_{regs} in cervical cancer patients.

Table 2. Effect of CD25$^+$ cell depletion on interferon (IFN)-γ T cell responses against HPV16 E6 and/or E7 in cervical cancer patients and healthy controls.

	PBMC			CD 25 depleted PBMC		
Cervical cancer patients	E6	E7	MRM	E6	E7	MRM
HPV16 pos						
Patient 1	9	3	**38**	9	**10**	**88**
Patient 2	0	0	**31**	0	9	**37**
Patient 3	0	1	**> 400**	0	6	**> 400**
Patient 7	0	0	**170**	0	0	**91**
Patient 8	3	2	**12**	**42**	**16**	**74**
Patient 12	**12**	4	**39**	**43**	**29**	**56**
Patient 14	1	3	**38**	7	**10**	**76**
Patient 15	0	0	**45**	2	0	**56**
Patient 19	0	1	**39**	4	3	**157**
Patient 21	7	3	**> 400**	**14**	7	**> 400**
HPV16 neg						
Patient 5	0	0	**> 400**	0	0	**> 400**
Patient 6	0	1	6	0	4	**95**
Patient 10	0	0	0	9	0	4
Patient 11	0	0	**102**	0	0	**167**
Patient 16	1	1	**28**	0	1	**38**
Controls						
Control 1	0	1	**77**	0	0	**112**
Control 2	1	1	**65**	0	3	**80**
Control 3	0	1	**170**	0	2	**51**
Control 4	0	0	5	0	0	**85**
Control 5	0	0	**170**	0	0	120
Control 6	0	1	**43**	0	0	**118**
Control 7	0	**10**	**110**	0	0	**159**
Control 8	0	2	**49**	X	X	X
Control 9	0	0	**78**	**21**	0	**79**
Control 10	0	0	**> 400**	6	0	**269**

HPV16 E6- and E7-specific T cell responses in patients with cervical cancer and controls as determined by interferon (IFN)-γ enzyme-linked immunospot. The table shows the number of spots per 1×10^5 cells. Shown are the responses in total peripheral blood mononuclear cells (PBMC) and the CD25-depleted PBMC. Specific responses ($\geq 10/1 \times 10^5$ cells) are indicated in bold type. Analyses were performed with PBMC collected before invasive therapy. MRM: memory recall mix, E6: E6 peptide pool, E7: E7 peptide pool, X: insufficient cells available for analysis.

Table 3. Effect of CD25$^+$ cell depletion on antigen-specific interferon (IFN)-γ responses against human papilloma virus (HPV)16 E6 and/or E7 in both CD4$^+$ and CD8$^+$ T cell subsets of HPV16$^+$ cervical cancer patients.

	PBMC			CD25 depleted PBMC		
Cervical cancer patients	Neg control	E6 pept	E7 pept	Neg control	E6 pept	E7 pept
% IFN-γ + CD4+ T cells						
Patient 2	0·9	**3·4**	**3·6**	1	**7·5**	**4·8**
Patient 4	2·3	**7**	2·9	2·8	**9·2**	n.a.
Patient 20	0·4	**2·3**	0·6	0·4	**4·5**	**1·6**
Patient 22	0·8	**7·1**	**1·7**	1·2	**7·8**	1·4
%IFN-γ + CD8+ T cells						
Patient 2	0·5	**1·4**	0·8	0·3	**6·3**	**0·8**
Patient 4	0·8	**4·1**	**1·9**	n.a.	n.a.	n.a.
Patient 20	0·4	**2·1**	0·6	0·7	**1·7**	1
Patient 22	0·5	0·7	0·3	0·3	0·6	0·3

Human papilloma virus (HPV)16 E6 and E7-specific interferon (IFN)-γ T cell production was measured by intracellular cytokine staining after *in vitro* expansion for 12 days with peripheral blood mononuclear cells (PBMC) or CD25-depleted PBMC of four HPV16 + cervical cancer patients in the presence of HPV16 E6 or E7 peptide pools. The results are expressed as percentage IFN-γ positive cells within the CD4$^+$ or CD8$^+$ T cell fraction. Positive responses (at least two times the background) are indicated in bold type. Analyses were performed with PBMC obtained before invasive therapy. E6 pept: E6 peptide pool, E7 pept: E7 peptide pool, n.a. insufficient cells available for analysis.

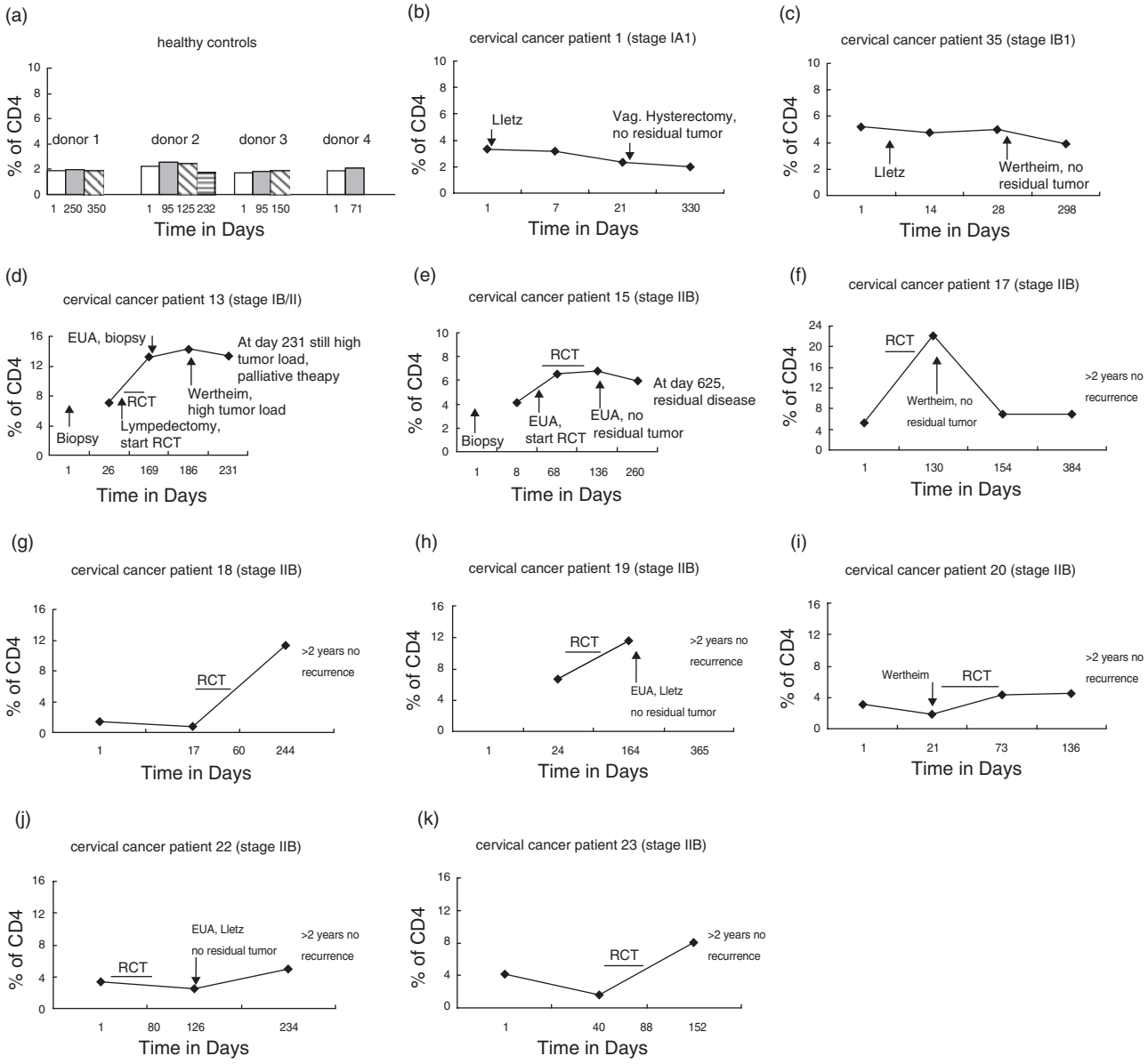

Fig. 4. The figure shows the longitudinal $CD4^+/CD25^{high}$ T cell frequencies as a percentage of the $CD4^+$ T cell fraction in four healthy controls (a) and 10 cervical cancer patients (c–k) during and following their therapy. Type of intervention/therapy and remarks about clinical status is mentioned in the figure. EUA: examination under anaesthesia; RCT: radiotherapy combined with chemotherapy.

Our results regarding suppression of HPV16 E6- and E7-specific T cell responses by $CD25^+$ T cells shed new light on observations of relatively low or impaired HPV-specific cellular immunity in cervical cancer patients [17–23]. In this study we provide evidence that intrinsically these anti-HPV responses exist, but are suppressed by T_{regs}. Therefore, the observed (low) level of HPV-specific immunity in PBMC of cervical cancer patients [17–23] is most probably an underestimation of the actual HPV-specific T cell numbers.

Malignant cervical tumour cells can produce large amounts of transforming growth factor (TGF)-β [34]. Because TGF-β induces T_{reg} development [35], this might be one of the mechanisms leading to increased T_{reg} levels in cervical cancer patients. However, theoretically, it cannot be ruled out that women developing CIN or cervical cancer display intrinsically increased T_{reg} numbers. If the increased T_{reg} numbers are induced by the cervical tumour only, tumour eradication might lead to a decline in T_{reg} numbers. However, we found only a modest decline of $CD4^+/CD25^{high}$ T cell frequencies in the year following curative surgery. Patients treated with chemoradiation showed strong fluctuations but no decrease of their $CD4^+/CD25^{high}$ T cell frequencies during and following therapy. Chemoradiation causes tissue damage and apoptosis which may lead to immune activation. As a consequence the number of T_{regs} might be up-regulated to prevent immune pathology [1–4]. It remains

an unanswered question as to whether T_{regs} are induced or already present in patients with (pre)malignant cervical neoplasia. Therefore, further research will be required to elucidate fully the influence of therapy on T_{regs} in these patients.

Because E6 and E7 transforming oncoproteins are crucial for transformation and maintenance of the malignant phenotype, they are ideal candidates for tumour-specific cervical cancer immunotherapy [36]. The results presented in this study indicate that the presence of increased T_{reg} numbers is another hurdle to overcome for successful therapeutic immunotherapy, especially when this therapy is given as an adjuvant to chemoradiation. Therefore, immunization strategies should elicit strong anti-tumour immune responses: strong enough to overcome the immunosuppressive state of the patient. Such strong responses have been described for immunizations with a genetic vector derived from Semliki-Forest virus (an alphavirus) expressing HPV16 E6/E7, which could break immune tolerance in HPV-transgenic mice [37].

Another approach could be (temporarily) bypassing suppression of cellular immunity by T_{regs} during therapeutic immunizations for treatment of (pre)malignant cervical neoplasia. The clinical potential of enhancing anti-tumour immune responses by T_{reg} depletion has been shown recently in patients with metastatic renal carcinoma [38]. However, interfering in T_{reg}-controlled immune responses should be conducted with extreme caution, because of the risk of developing autoimmunity [39].

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