Ovarian cancer cytoreduction induces changes in T cell population subsets reducing immunosuppression

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Abstract

Surgery is the primary therapeutic strategy for most solid tumours; however, modern oncology has established that neoplasms are frequently systemic diseases. Being however a local treatment, the mechanisms through which surgery plays its systemic role remain unknown. We have investigated the influence of cytoreduction on the immune system of primary and recurrent ovarian cancer. All ovarian cancer patients show an increase in $CD4^+CD25^+FOXP3^+$ circulating cells (CD4 T_{reg}). CD4/CD8 ratio is increased in primary tumours, but not in recurrent neoplasms. Primary cytoreduction is able to increase circulating CD4 and CD8 effector cells and decrease CD4 naïve T cells. $CD4^+$ T_{reg} cells rapidly decreased after primary tumour debulking, while $CD8^+CD25^+FOXP3^+$ (CD8 T_{reg}) cells are not detectable in peripheral blood. Similar results on CD4 T_{reg} were observed with chemical debulking in women subjected to neoadjuvant chemotherapy. CD4 and CD8 T_{reg} cells are both present in neoplastic tissue. Interleukin (IL)-10 serum levels decrease after surgery, while no changes are observed in transforming growth factor-β_1 and IL-6 levels. Surgically induced reduction of the immunosuppressive environment results in an increased capacity of $CD8^+$ T cells to respond to the recall antigens. None of these changes was observed in patients previously subjected to chemotherapy or affected by recurrent disease. In conclusion, we demonstrate in ovarian cancer that primary debulking is associated with a reduction of circulating T_{reg} and an increase in CD8 T-cell function. Debulking plays a beneficial systemic effect by reverting immunosuppression and restoring immunological fitness.

Keywords: interval debulking surgery ● ovarian cancer ● primary cytoreduction ● secondary cytoreduction ● T_{reg} cells

Introduction

Ovarian cancer is the most lethal gynaecological cancer with over 15,000 estimated deaths in United States in 2008 [1]. Standard treatment is primary cytoreduction followed by adjuvant platinum and taxane based chemotherapy [2–4]. An alternative therapeutic strategy under investigation is neoadjuvant chemotherapy (NACT) followed by interval debulking surgery (IDS) and adjuvant chemotherapy [5–7]. Patients' prognosis is strictly dependent on surgical outcome [6–8]. This has lead research worldwide to propose cytoreduction also for the treatment of platinum sensitive recurrent disease (secondary cytoreduction) [9].

There are several hypotheses that could explain the important clinical impact of cytoreductive surgery in this cancer. It is reasonable to believe that surgery is able to remove poorly vascularized tissues, decrease tumour burden and therefore allow proliferation of tumour residual cells that are more susceptible to cytotoxic drugs [8, 10, 11]. Recently, an appealing hypothesis that is emerging is that tumour debulking reduces tumour-induced immunosuppression [12].

It is well known that neoplasms are able to induce immune tolerance through different mechanisms including the release of immune suppressive cytokines (interleukin [IL]-10, transforming growth factor [TGF]-β_1, VEGF, prostaglandin E2 [PGE_2]) [11, 13, 14], deletion of tumour-reactive T cells [15], induction of suppressive

or dysfunctional antigen presenting cells (APC) [15]. In the last decade, the fundamental role of naturally or induced T_{reg} cells in tumour progression has been demonstrated. T_{reg} cells are able to maintain immune tolerance, thanks to their ability to inhibit CD4 and CD8 T lymphocyte activation [16]. In women affected by epithelial ovarian malignancies, T_{reg} are recruited primarily in neoplastic tissue and ascites [17]. The detrimental effect of T_{reg} cells and their inhibitory activity on cytotoxic lymphocytes are well established by the strong prognostic correlation of CD8/CD4 T_{reg} ratio [18]. Considering the numerous immunosuppressive mechanisms adopted by cancer cells, it is conceivable to believe that tumour removal could affect the immunological repertoire of cancer patients, but, currently, no clinical data are available to support this hypothesis [19].

In this study, we analysed the effect of tumour debulking in ovarian cancer patients' immune signatures, in order to understand if immunological mechanisms might be involved in the clinical benefit associated with the achievement of primary optimal residual disease.

Materials and methods

Patients' characteristics

This study was approved by the institution IRB and by the ethical committee of the University of Rome 'Sapienza', and informed consent was obtained by all patients. Patients subjected to primary, IDS or secondary cytoreduction were recruited from the Department of Gynecology and Obstetrics of the University of Rome 'Sapienza'. Primary cytoreduction and IDS consisted in hysterectomy with bilateral salpingo-oophorectomy, omentectomy, appendectomy, extensive peritoneal stripping including the diaphragm, bowel and liver resection and systematic lymphadenectomy, when necessary. Secondary cytoreduction consisted in removal of all visible tumour carrying out one or more procedures described above. All patients were treated with carboplatin-based chemotherapy. In particular, patients in the IDS group were subjected to exploratory surgery and received three cycles of NACT with carboplatin and paclitaxel before surgical cytoreduction. The reported analyses were carried out on patients who achieved no visible tumour after primary, IDS or secondary cytoreduction. Blood and serum samples were collected the day before surgery (day 0), between 2 and 4 days after surgery (day 2) and proximally at the 2 weeks post-operative office control before initiating any adjuvant treatment (day 15). Samples were also collected before exploratory surgery (ES) in the IDS group. Patients with visible tumour after surgery or with severe post-operative complications (*e.g.* post-operative severe infection or thromboembolisms) were excluded from the present analyses. Patients with severe morbidities and in treatment with immune suppressive drugs or history of other neoplasms were also not considered. Controls were collected from patients with no history of cancer, subjected to laparotomy for benign gynaecological conditions. Histological typing and grading were reviewed with World Health Organization criteria and surgical stage was assigned using the classification system of the International Federation of Gynecology and Obstetrics. Patients were analysed as four groups: women with diagnosis of primary epithelial ovarian cancer (pOC: primary ovarian

cancer), patients subjected to IDS, patients with diagnosis of recurrent epithelial ovarian cancer (rOC: recurrent ovarian cancer) and controls.

Cell line

HLA-A*0201 transfected K562 cell line was cultured in RPMI 1640 + GlutaMAX (Gibco, Invitrogen, USA), 1% penicillin/streptomycin (Hyclone, USA), 10% foetal calf serum (Hyclone, Waltham, MA, USA) and G418 (1 mg/ml) (Gibco, New York, NY, USA) at 37°C and 5% CO_2.

Peripheral blood mononuclear cell purification

Peripheral blood mononuclear cells (PBMCs) of 25 pOC patients, 25 patients treated with IDS, 25 patients affected by platinum sensitive recurrent epithelial ovarian cancer and 25 patients with benign gynaecological condition were collected at days 0, 2 and 15 after surgery. Samples were also collected at ES in the IDS group. PBMCs were isolated from 10–12 ml of blood by Ficoll-Hypaque gradient (1077 g/ml; Pharmacia LKB, Uppsala, Sweden). The purified PBMCs were counted obtaining a yield between 10×10^6 and 12×10^6 cells for each drawing and criopreserved until used.

Flow cytometry

Cell phenotype staining was performed using the following panel of mouse monoclonal antibodies (MoAbs): anti-CD4-FITC and -PE-Cy5 (IgG_1; RPA-T4), anti-CD3-PE (IgG_1; UCHT1), anti-CD45RA-APC (IgG_{2b}; HI100), anti-CD8-PE-Cy5 (IgG_1; RPA-T8), anti-CD25-PE (IgG_{2a}; 24212) and anti-FOXP3-Alexa647 (IgG_1; 259D/C7) (BD Pharmingen, Franklin Lakes, NJ, USA) and anti-CCR7-FITC (IgG_{2a}; 150503) (R&D Systems, Minneapolis, MN, USA). Cells were analysed on FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA), running Cell Quest data acquisition and analysis software (Becton Dickinson). To determine the percentage of CD4 and CD8 T_{reg} cells, lymphocytes were gated by plotting forward scatter *versus* side scatter, followed by gating of the $CD4^+CD25^+$ or $CD8^+CD25^+$ cells. The expression of FOXP3 was evaluated on $1–2 \times 10^3$ $CD4^+CD25^+$ or $CD8^+CD25^+$ cells.

Immunosuppression assay

$CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ T cells were cultured alone or co-cultured at two different ratios ($CD4^+CD25^{high}/CD4^+CD25^-$, 1:10 and 1:1) with 1 μg/ml of anti-CD3 (X35; Immunotech, Paris, France) and 5 μg/ml of anti-CD28 (CD28.2; BD Pharmingen) antibodies. Proliferation was measured by [^{3}H]thymidine (1 μCi (0.037 MBq) per well) (Perkin-Elmer, Waltham, MA, USA) incorporation pulsed on day 4 and quantified 18 hrs later using a liquid scintillation counter (Perkin-Elmer). All experiments were done in triplicate wells. One hundred percent proliferation was defined as the proliferation of $CD4^+CD25^-$ T cells without co-culturing with T_{reg} cells.

Serum cytokine detection

The levels of IL-10, TGF-β_1 and IL-6 were measured in patients' sera by ELISA kits purchased from R&D Systems, DRG Diagnostic (Marburg, Germany) and Pierce Endogen (Rockford, IL, USA), respectively.

Confocal microscopy

Formalin-fixed, paraffin-embedded samples of two pOC, two IDS and two rOC patients were deparaffinized and underwent antigen retrieval with citrate buffer (pH 6.0). Slides were incubated with anti-CD4-FITC (1:30) (IgG$_1$; RPA-T4) (BD Pharmingen) or anti-CD8-FITC (1:30) (IgG$_{2b}$; 37006) (R&D Systems) and with anti-FOXP3-Alexa647 (IgG$_1$; 259D/C7) (BD Pharmingen) (1:30), for 1 hr at RT. Imaging was performed by two-photon absorption fluorescence with the confocal laser-scanning microscope C1Nikon Plus excited by a Ti:sapphire ultrafast laser source (Mai Tai Laser 750–850, Spectra Physics, Santa Clara, CA, USA). Co-localization was performed using SVI software (Scientific Volume Imaging, Hilversum, The Netherlands).

T-cell stimulation and IFN-γ ELISpot assay

PBMCs, derived from three pOC patients at days 0 and 15, were co-cultured with HLA-A2$^+$K562 cell line, pulsed with Flu peptide (GILGFVFTL, 10 mg/ml) (ProImmune, Oxford, UK) and irradiated with 30 Gy, for 12 days in presence of IL-2 (10 U/ml) and IL-15 (10 ng/ml) (R&D Systems). At day 12, CD8 T cells were purified and plated in triplicate for 24 hrs with transfected K562 cells with or without Flu peptide (50 mg/ml). Interferon (IFN)-γ production was detected by ELISpot assay. Spots were counted using the ImmunoSpot Image Analyzer (Aelvis, Cologne, Germany).

Statistical analysis

Descriptive statistics (average and S.D.) were used to describe all various groups of data. Parametric tests were used after having evaluated the normal distribution of the data to be analysed. In particular, the Student's two-tailed t-test for paired and unpaired data was employed. The Fisher's exact test and the χ^2-test were used for categorical data where appropriate. Multiple comparisons were evaluated by analysis of variance and any significant difference was identified using the Bonferroni correction for multiple comparisons. Statistical significance was set at a P-value less than 0.05.

Results

Patients' characteristics

Patients' characteristics are described in Table 1. Briefly, pOC patients were mostly stage IIIC papillary serous highly undifferentiated ovarian neoplasms. IDS patients were predominantly stage III papillary serous and mucinous histotype. All patients subjected to IDS had a partial clinical response, with 19 having a CA125 reduction of over 50% [20]. Definitive pathological report of the IDS showed multiple persistent abdominal and pelvic lesions of over 2 cm in greatest size in all patients. rOC patients were platinum sensitive ovarian cancer women with isolated or multiple lesions, but with no carcinomatosis. The majority of women had retroperitoneal disease. The control group was mostly represented by patients subjected to hysterectomy with or without prophylactic bilateral adnexectomy.

Table 1 Patients' characteristics

Ovarian cancer		Recurrent ovarian cancer	
No of patients	25	No of patients	25
Mean age (range)	58 (33–69)	Mean age (range)	66 (44–69)
FIGO stage		Grading	
III	23	1	4
IV (hepatic)	2	2	2
Grading		3	19
1	1	Histotype	
2	2	Papillary serous	17
3	22	Mucinous	7
Histotype		Endometroid	1
Papillary serous	16	Disease-free survival	
Mucinous	4	<12 months	1
Clear cell	1	>24 months	16
Endometroid	4	12–24 months	8
		Location of recurrence*	
		Lymph node only	14
		Intra-peritoneal with or w/o nodal involvement	11
Interval debulking disease		Control	
No of patients	25	No of patients	25
Mean age (range)	57 (44–72)	Mean age (range)	52 (24–72)
FIGO stage		Surgical procedures	
III	24	Hysterectomy ± adnexectomy	15
IV (hepatic)	1	Adnexectomy	4
CA125 < 50%	19	Myomectomy	6
CA125 > 50%	6		
Grading			
1	3		
2	5		
3	17		
Histotype			
Papillary serous	18		
Mucinous	7		

*More than one per patient.

Table 2 Circulating T-cell population

	% CD8 T cells[§]		% CD4 T cells		% CD3**	
	Day 0	Day 15	Day 0	Day 15	Day 0	Day 15
pOC	20 ± 6	19 ± 8	48 ± 10*	38 ± 17*	43 ± 9	43 ± 7
IDS	21 ± 3.5	20 ± 7.8	51 ± 8	43 ± 19	44.5 ± 7	47 ± 3.5
rOC	23 ± 5	24 ± 7	45 ± 8	40 ± 15	47 ± 4	42 ± 9
Control	25 ± 9.6	22 ± 7	46 ± 8	47 ± 10	43 ± 10	42 ± 5

[§]All values are reported as mean percentage $\pm$ S.D.

**The percentage of CD3 is calculated on the entire PBMC population, while the percentage of CD8 and CD4 T cells are estimated on CD3+ cells.

*Percentage of CD4 T cells was significantly decreased after primary cytoreduction $P < 0.05$.

Primary cytoreduction affects circulating T cell population inducing a rapid decrease of CD4 cells, mainly of the T_{reg} subset

The proportion of the circulating T-cell population and the relative fractions of $CD3^+CD8^+$ and $CD3^+CD4^+$ were analysed in the four groups at days 0 and 15 and results are reported in Table 2. The percentage of CD8 T cells before surgery was similar in all groups and no significant modifications could be observed in the post-operative period. In patients subjected to primary cytoreduction, there was a significant decrease in the percentage of CD4 T cells after 2 weeks from surgery ($P < 0.05$). The fractions of $CD3^+$ cells in the four groups were comparable at all time-points. The CD4/CD8 ratio was significantly higher in pOC and IDS patients compared to control (pOC *versus* control 2.6 ± 1.2 *versus* 2.0 ± 0.8, $P < 0.05$; IDS *versus* control 2.6 ± 0.8 *versus* 2.0 ± 0.8, $P < 0.05$) before surgery. These differences were no longer present 2 weeks after primary cytoreduction. In rOC groups, the CD4/CD8 ratio did not change significantly after surgery and remained similar to the control.

In order to evaluate the proportion of T_{reg} cells and the changes induced by tumour removal, T_{reg} population was identified by co-expression of CD4, CD25 and FOXP3 markers and by immunosuppression assay.

Figure 1A shows the progressive reduction of $CD4^+CD25^+$ T-cell population and the relative expression of FOXP3 before, immediately and 2 weeks after primary cytoreduction in a representative patient affected by Federation Internationale de Gynecologie et d'Obstetrique (FIGO) stage IIIC. The percentage of $CD4^+CD25^+$ T cells decreased from 2.5% to 1.8% to 1.6% at days 0, 2 and 15, respectively. Furthermore, the mean fluorescence intensity decreased in the three consecutive samples.

The phenotype analysis carried out on all patients (Fig. 1B) showed that at day 0 $CD4^+CD25^+FOXP3^+$ cells were significantly higher in the pOC group as compared to the control. Mean percentage of T_{reg} cells rapidly decreased from $2.4 \pm 1.2\%$ to $1.7 \pm 0.9\%$ ($P < 0.01$) after two days from surgery. The decline in T_{reg} cells continued between days 2 and 15 (from $1.7 \pm 0.9\%$ to $1.5 \pm 1\%$; $P < 0.05$). Although this rapid and significant reduc-

tion observed in the pOC group, the T_{reg} cells overall percentage remained higher than the one present in the control group (pOC $1.5 \pm 1\%$ *versus* control $0.3 \pm 0.1\%$; $P < 0.0005$). In IDS and rOC patients the relative percentage of T_{reg} cells was always significantly higher than the control. T_{reg} cell levels did not vary in both groups in the three time-points analysed.

In order to monitor the immunosuppression capability of $CD4^+CD25^{high}$ ($FOXP3^+$) T_{reg} cells, three pOC patients' cell samples were tested for their ability to inhibit $CD4^+CD25^-$ T-cell proliferation upon stimulation with anti-CD3 and anti-CD28 monoclonal antibodies. Figure 1C shows the results of a representative donor. T_{reg} cells exhibit different level of suppressive activity when they are used at different ratios. This ability corresponds to 66% when the proportion of suppressors/effectors is 1:1, but decreases at 10% at 1:10 ratio.

Collected blood samples were also tested to identify the presence of circulating CD8 T_{reg} population ($CD8^+CD25^+FOXP3^+$) and their variability after surgery. Figure 2A shows the presence of $CD8^+CD25^+$ cells in the peripheral blood at all time-points analysed, but this population failed to express the FOXP3 marker.

In addition, the presence of tumour infiltrating regulatory lymphocytes in ovarian cancer neoplasm was evaluated by confocal microscopy. Figure 2B and C shows the results of tumour infiltrating $CD4^+FOXP3^+$ and $CD8^+FOXP3^+$ cells within the neoplastic tissue of a representative pOC patient. CD4 (B) or CD8 (C) markers are visualized in green in the first column, while FOXP3 molecule is in red in the second column. Co-localization areas (in white) and phase contrast are shown in the third and fourth columns, respectively. $CD4^+$ and $CD8^+$ cells were both detected in the neoplastic tissue. Importantly, some of the $CD4^+$ and $CD8^+$ cells were also positive for FOXP3 molecule showing that not only $CD4^+$ T_{reg}, but also $CD8^+$ T_{reg} are detectable in tumour. Lymphocyte infiltrating patterns were similar in the three different ovarian cancer populations.

Primary cytoreduction affects the immunological repertoire of circulating effector T lymphocytes

CD4 and CD8 T-cell populations were analysed by cytofluorimetry for the expression of CD45RA and CCR7 markers to evaluate
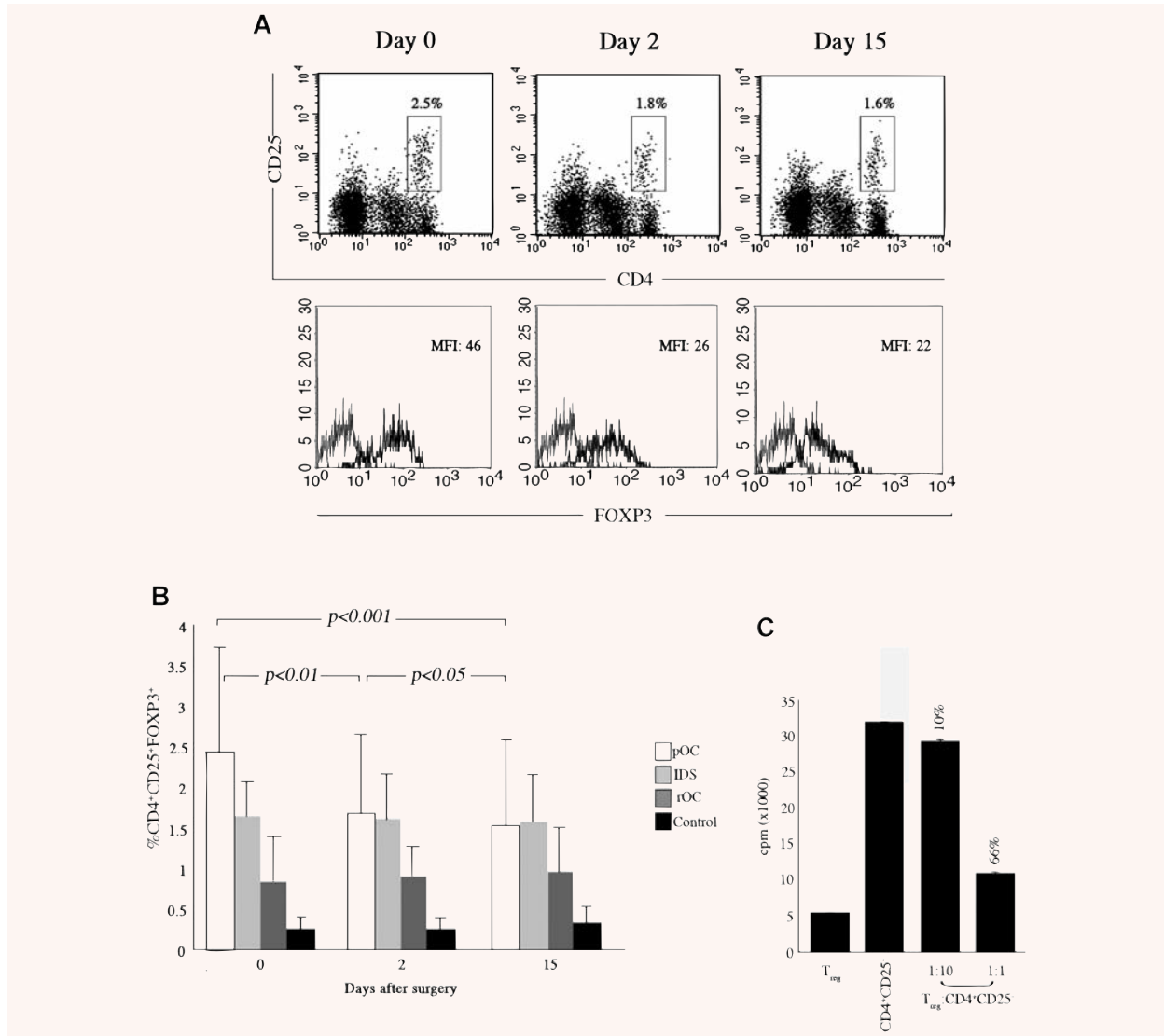
Fig. 1 Circulating CD4 T_{reg} cell analysis in ovarian cancer patients and controls and T_{reg} immunosuppression assay. Lymphocytes were analysed by cytofluorimetry for the expression of CD4, CD25 and FOXP3 molecules. (**A**) shows dot plots of CD4 and CD25 markers after gating on lymphocytes at days 0, 2 and 15 (in the square, the percentage of double positive cells). Histogram plots represent the expression of FOXP3 in $CD4^+CD25^+$ T cells with the corresponding mean fluorescence intensity (bold line and thin line corresponding to FOXP3 antigen and isotype control, respectively). Results are related to a representative pOC donor. (**B**) shows $CD4^+CD25^+FOXP3^+$ cells in pOC (white columns), IDS (light grey column) and rOC (dark grey column) patients compared to controls (black columns) at days 0, 2 and 15. Data are reported as mean percentage of $CD4^+CD25^+FOXP3^+$ cells $\pm$ S.D. (**C**) represents the suppression ability of purified $CD4^+CD25^{high}T_{reg}$ cells when co-cultured with $CD4^+CD25^-$ T cells at two different ratios ($CD4^+CD25^{high}/CD4^+CD25^- = 1:10$ and 1:1) upon stimulation with anti-CD3 and anti-CD28 antibodies. Experiments were done in triplicate in three different pOC patients before surgery and the average values of cpm (counts per minute) of one representative donor out of three were plotted. In high, the percentage values of immunosuppression are reported.

the proportion of naïve ($CD45RA^+CCR7^+$), central memory ($CD45RA^-CCR7^+$), effector memory ($CD45RA^-CCR7^-$) and terminally differentiated effector ($CD45RA^+CCR7^-$) subsets [21] (Fig. 3). Figure 3A shows the changes of circulating CD4 T cell after tumour removal. Before surgery, the relative proportion of

CD4 effector T cells was significantly higher ($P < 0.05$) in pOC patients when compared with the control group. There was a significant increase in CD4 effector T cells after surgery ($P < 0.05$). Before surgery, CD4 naïve T cells were significantly lower in the pOC group as compared to controls. In the pOC
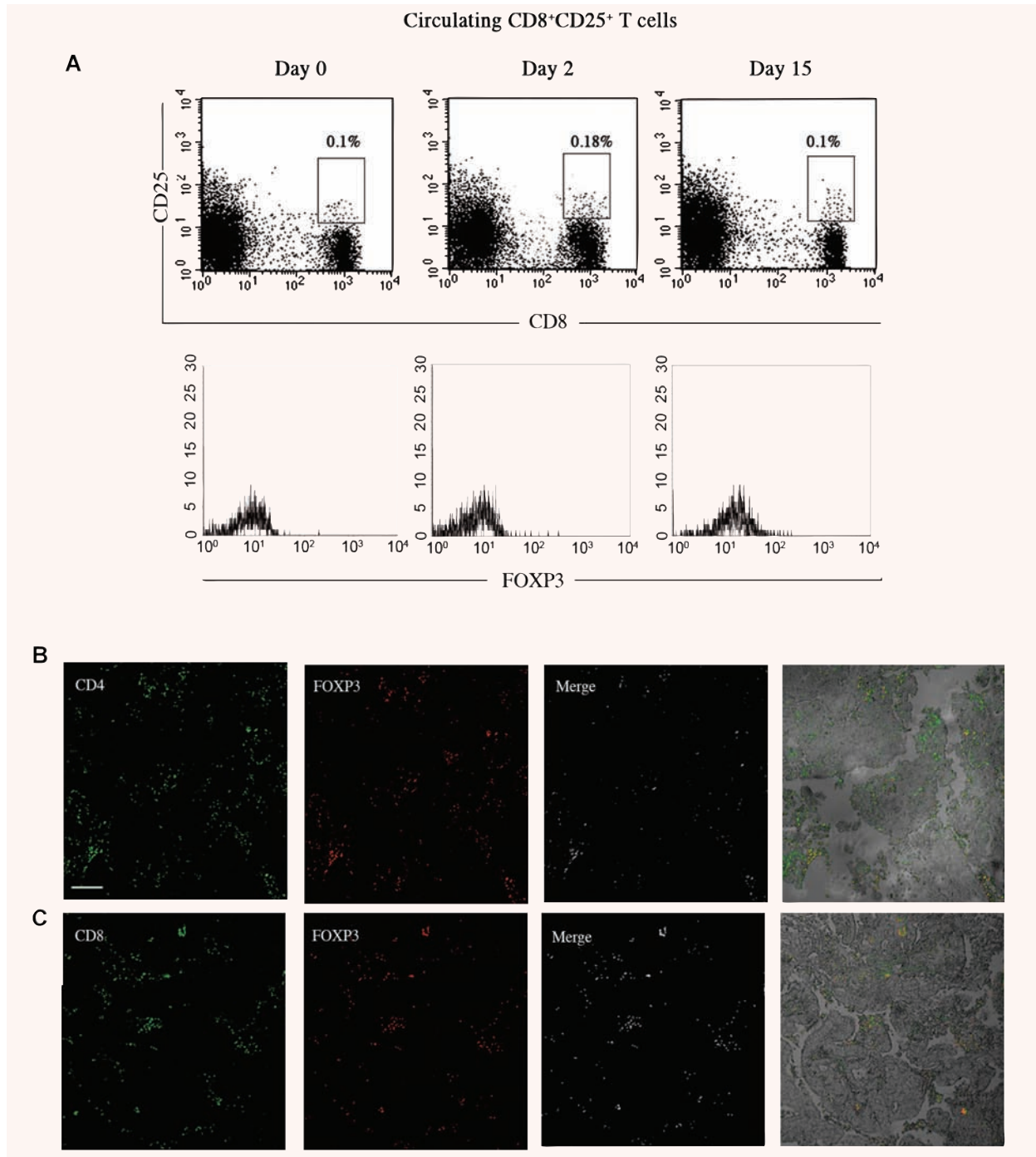
Fig. 2 CD8$^+$CD25$^+$ T-cell analysis in peripheral blood and CD4 and CD8 T$_{reg}$ cell analysis in tumour. (**A**) shows dot plots of CD8 and CD25 markers after gating on lymphocytes at days 0, 2 and 15 (in the square, the percentage of double positive cells). The expression of FOXP3 in CD8$^+$CD25$^+$ T cells is showed in the histogram plots (bold line and thin line corresponding to FOXP3 antigen and isotype control, respectively). Results are related to a representative pOC donor. (**B**) and (**C**) show confocal microscopy of CD4$^+$FOXP3$^+$ and CD8$^+$FOXP3$^+$ cells in ovarian cancer tissue of a representative pOC patient. Paraffin-embedded pOC tissue was stained with anti-FOXP3 and anti-CD4 or anti-CD8 antibodies. In the first column are visualized in green CD4 (**B**) and CD8 (**C**) markers, while FOXP3 molecule is in red in the second column. The last two columns show the co-localization areas and the contrast-phase images, respectively. Magnification:10×; Bar: 25 μm.
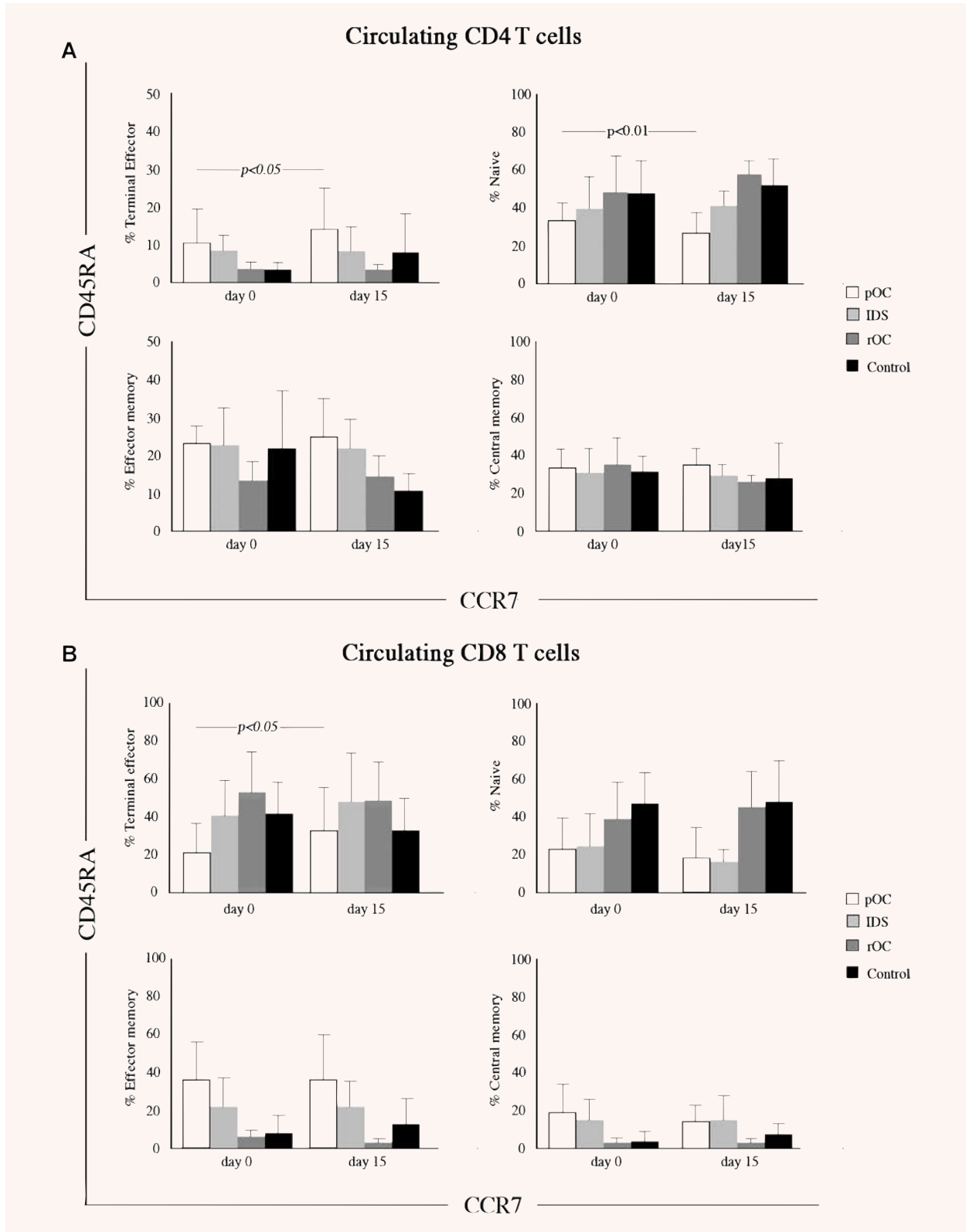
Circulating CD4 T cells

Circulating CD8 T cells

Fig. 3 CD4 and CD8 T-cell subsets in pOC (white column), IDS (light grey column) and rOC (dark grey column) patients compared to control (black column), at days 0 and 15. PBMCs were analysed by cytofluorimetry after cell surface labelling with anti-CCR7, anti-CD45RA, anti-CD3 and anti-CD4 or anti-CD8 antibodies. The analysis were performed on $CD3^+CD4^+$ (**A**) and $CD3^+CD8^+$ (**B**) populations after gating on lymphocytes. $CD45RA^+CCR7^+$, $CD45RA^-CCR7^+$, $CD45RA^-CCR7^-$, $CD45RA^+CCR7^-$ corresponding to naïve, central memory, effector memory and terminally differentiated effector cells, respectively. Data are reported as mean of percentage of $CD3^+CD4^+$ or $CD3^+CD8^+$ cells $\pm$ S.D.

group, there was a significant decrease in CD4 naïve T cells after surgery ($P < 0.01$).

At day 0, IDS patients had a higher percentage of CD4 effector T cells than the control, but no changes occurred after tumour removal. In this group the other CD4 populations were similar to the control group and did not vary after tumour debulking.

rOC patients showed a T-cell population pattern similar to the control group and no significant modifications were noted after secondary cytoreduction.

Circulating CD8 T cells were also analysed in order to evaluate the proportion of the different CD8 T-cell subsets (Fig. 3B). Before surgery, pOC patients had a significantly lower proportion of CD8 effector T cells and CD8 naïve T cells compared to controls, whereas CD8 central memory and effector memory T cells were significantly higher. Tumour primary debulking induced a significant increase in CD8 effector T cells ($P < 0.05$), while no changes were observed in the other T-cell subsets.

IDS patients had a significantly lower proportion of naïve T cells and a higher level of CD8 memory and effector memory T cells than the control, but no modification occurred in all CD8 subsets after 2 weeks from surgery.

Similar to what observed in the CD4 population, no significant differences in the CD8 T subsets was observed between rOC patients and the control group. CD8 T-cell population did not vary significantly after secondary debulking.

CD8/CD4 T_{reg} ratio in tumour samples represents a significant prognostic factor [18]. pOC patients after tumour debulking had a significant increase in the ratio of circulating CD8/CD4 T_{reg} (8.5 $\pm$ 3.0 at day 0 to 13.0 $\pm$ 7.0 at day 15; $P < 0.05$). No variations were observed in the other three groups.

Neoadjuvant chemotherapy decreases the percentage of circulating T_{reg} cells

In the IDS group, the phenotype of lymphocyte populations derived from patients subjected to NACT were also evaluated at ES and compared with day 0 of pOC and IDS groups. The percentage of T_{reg} cells before chemotherapy was similar to the one observed at day 0 in the pOC group (day 0 pOC *versus* ES 2.4 $\pm$ 1.2 *versus* 2.9 $\pm$ 1.3, $P = 0.3$) (Fig. 4A). T_{reg} cells significantly decreased after three cycles of chemotherapy (ES *versus* day 0 IDS 2.9 $\pm$ 1.3 *versus* 1.6 $\pm$ 0.5, $P < 0.005$). No difference between CD4 and CD8 T lymphocyte subsets at ES and at day 0 of the IDS group was present. These results suggest a role of NACT in the reduction of T_{reg} cells, but no modifications in the CD4 and CD8 subset are present (Fig. 4B and C).

IL-10 serum level is reduced after primary cytoreduction

Serum samples were collected in parallel to the PBMCs and were analysed for the concentration of IL-10, TGF-β_1 and IL-6 by ELISA. Data regarding patients affected by primary neoplasms and control are shown in Fig. 5A. Primary debulking induced a significant reduction in IL-10 concentration. TGF-β_1 and IL-6 were not affected by tumour debulking. IDS and rOC serum cytokine levels did not vary after surgery (IDS at day 0: IL-10, TGF-β_1, IL-6: 65 $\pm$ 17.5, 21 $\pm$ 6, 5 $\pm$ 3, respectively; rOC at day 0: IL-10, TGF-β_1, IL-6: 66 $\pm$ 15, 22 $\pm$ 5, 4.5 $\pm$ 3, respectively).

T cell capacity to respond to specific and unspecific antigens increases after primary cytoreduction

HLA-A*0201$^+$ PBMCs derived from pOC patients before and after surgery were stimulated for 12 days with Flu peptide in order to enrich the lymphocyte population of Flu specific CD8 T cells. After stimulation, CD8 T cells were purified and used as responders for IFN-γ ELISpot assays, while HLA-A*0201 transfected K562 cell line was used as APCs. Figure 5B and C shows the results of a representative pOC patient. CD8 T cells, purified from PBMCs before cytoreduction, are able to secrete IFN-γ after the addition of Flu peptide in presence of K562 cells, but this capacity is significantly increased after surgery (Fig. 5B). Furthermore, CD8 lymphocytes secrete IFN-γ in absence of Flu peptide due to the allogeneic stimulus of K562 cells (Fig. 5C). This production becomes stronger at day 15, but remains significantly lower when compared to IFN-γ secretion obtained with the Flu peptide.

Discussion

This study demonstrates that the immunological status of ovarian cancer patients is significantly affected by surgery and chemotherapy. Cancer immunosuppression is partially reversible once the cause is removed and acquired immunity is enhanced by tumour debulking. Furthermore the immunological positive effect of surgery in ovarian cancer patients is primarily present when cytoreduction is carried out as first therapeutic step.

Fig. 4 Analysis of lympho-cyte subsets in ES group. (**A**) shows the percentage of circulating CD4$^+$CD25$^+$ FOXP3$^+$ cells in patients treated with NACT before ES compared to those present in IDS and pOC at day 0. (**B**) and (**C**) represent the percentage of circulating naïve (T$_N$), central memory (T$_{CM}$), terminally effector (T$_{EF}$) and effector memory (T$_{EFMRA}$) CD4 and CD8 T cells, respectively. In all panels the results are plotted as percentage of positive cells of 25 patients $\pm$ S.D.
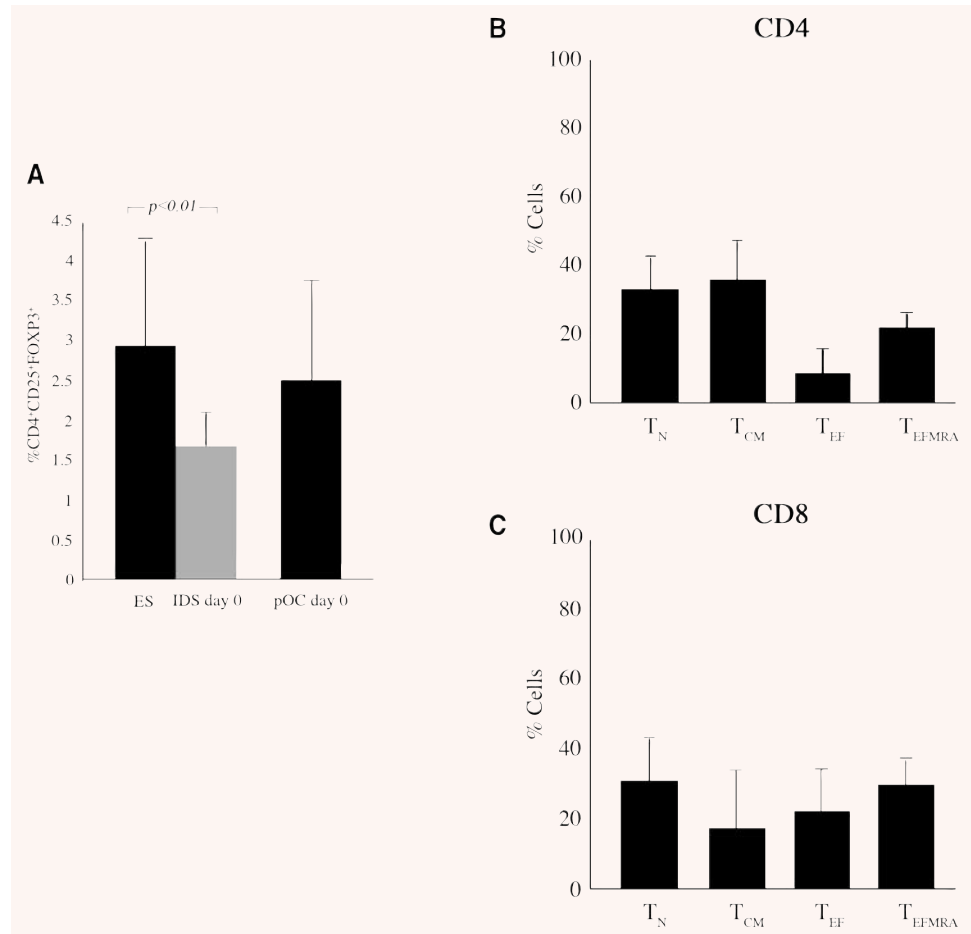
Recent data performed on mouse models have shown that removal of tumour-transplanted cells was associated with an increase in lymphocyte activation [22, 23]. Although essential and important for a preliminary understanding of the immunological mechanisms that cause tumour growth *in vivo*, these models are unable to examine the complex tumour–host interaction that is established during tumour progression (tumour editing), which includes the immunologic tumour cell selection process [24].

Tumour infiltrating lymphocytes are able to influence the patient's prognosis in ovarian as well as in other cancers. In particular, patients with intratumoral CD3$^+$ cells islets benefit from a significantly better prognosis [25]. Some authors have demonstrated that the phenotype of tumour infiltrating lym-phocytes is a better predictive factor of patients' outcome as compared to the sole absolute number [17]. Infiltrating T$_{reg}$ cells correlate with dismal prognosis [17] and CD8/CD4 T$_{reg}$ ratio is a reliable prognostic indicator [18]. Growing ovarian cancer appears to have the capacity to attract and activate large num-bers of T$_{reg}$ by a variety of mechanisms and therefore becomes rapidly a strong source of immunosuppressive signals [16, 17]. Very limited data are reported regarding the effects of this

phenomenon on the systemic immune system and on the circulating T cells.

Although a correlation between circulating and tumour infiltrat-ing cell has not yet been demonstrated, we have analysed the effect of cytoreduction on the systemic immune signature of women affected by ovarian cancer. Significant numbers of circu-lating T$_{reg}$ are consistently present in patients with clinically evident disease. Primary surgery induces a rapid decrease of these cells. This phenomenon is due to tumour burden removal because it is not observed in the control group. The consequence of cancer removal and T$_{reg}$ cell reduction is the decrease of the immunosuppressive status as demonstrated by the reduction of circulating IL-10. This physiopathological change in the immune signature of the patients is further suppressed by the significant decrease observed in the CD4/CD8 ratio. These changes ultimately result in a relative increase in systemic effector T-cell population. Interestingly T naïve are decreased. The opposite effect in terms of proliferation observed in naïve and effector lymphocytes could be explained by two different mechanisms. The elimination of sup-pression after tumour removal allows a new activation of naïve cells that generate new effectors. The increase of effector T cells
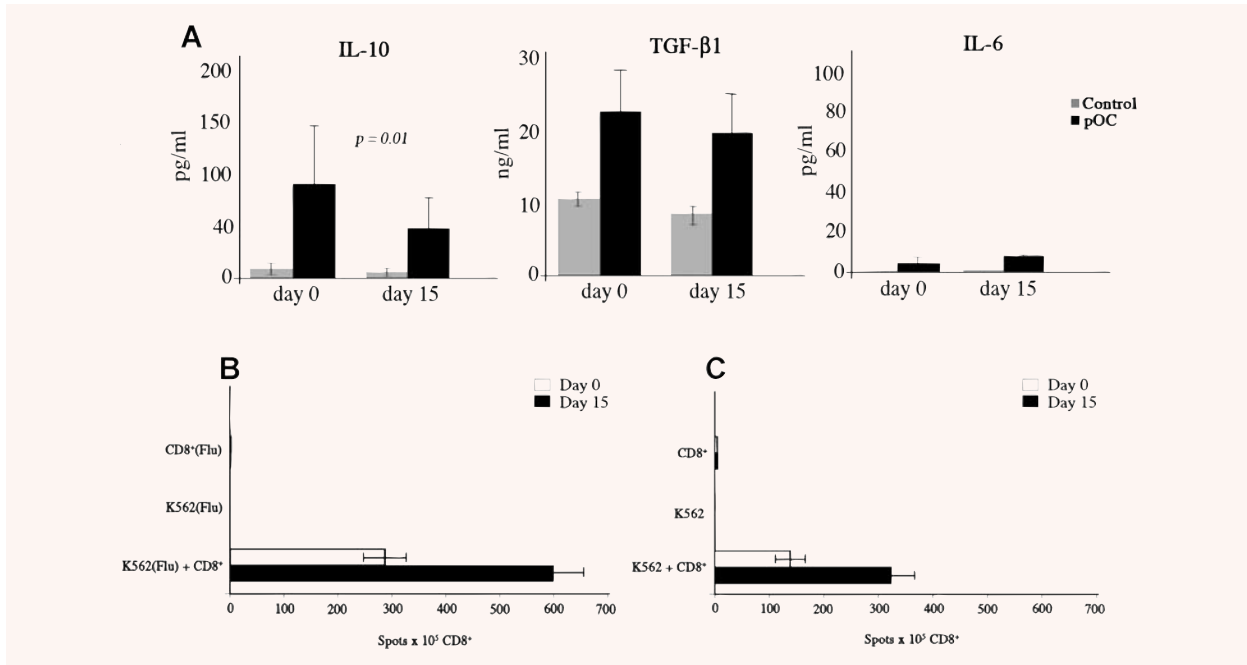
Fig. 5 IL-10, TGF-β_1 and IL-6 serum levels in pOC patients and control and ELISpot analysis of IFN-γ production of CD8 T cells. (**A**) The serum samples collected from all pOC (black) and benign (grey) patients at days 0 and 15 were analysed for cytokine concentration by ELISA. Results are reported as mean of serum cytokine concentrations (pg/ml or ng/ml) $\pm$ S.D. (**B** and **C**): ELISpot analysis of IFN-γ production of CD8 T cells stimulated with Flu peptide derived from one representative pOC patient before (white histogram) and after surgery (black histogram). Results are reported as mean values $\pm$ S.D. of IFN-γ spots produced by purified CD8 T lymphocytes added to K562 cells in presence (**B**) or in absence of Flu peptide (**C**). CD8 and K562 cells alone were used as control.

and the decrease of naïve cells could be considered as two correlated events. However, tumour debulking could also permit the clonal expansion of activated effector cells as an independent event. Anyhow, we demonstrate an overall increase in specific CD8 T-cell activity after tumour removal. Patients subjected to primary surgery are subsequently subjected to adjuvant chemotherapy consisting generally in six cycles of platin and taxane chemotherapy. A recent finding has shown how adjuvant chemotherapy is associated with a CD8^{+} T-cell functional recovery [26]. It is likely that the reduction of T_{reg} cells after cytoreduction might favour the immunological effect of chemotherapy by restoring immunological fitness.

In women subjected to NACT, a reduction of circulating T_{reg} cells and an increase in CD8 T effector cells was observed after medical treatments, demonstrating an immunological beneficial effect of chemical debulking. IDS did not influence the T-cell populations. The effect on the immune system of chemotherapy administered in different therapeutic moments of women with ovarian cancer is a major issue that remains to be explored in depth. Dedicated analyses are currently being carried out by our group to better address this subject. Even so, the results observed in the IDS group and especially the different behaviours observed in this population when compared to the pOC group, do allow to draw some

preliminary hypotheses. The observation that IDS does not diminish the percentage of CD4 T_{reg} cells could suggest that a limit to the immunoregulatory effects exists, so that once the maximal effect is reached (surgically or chemically), it cannot be modulated further. Another hypothesis could be based on the observation that certain chemotherapic agents are able to reduce immune suppression and activate an acquired immune response [27–30].

The absence of any successive immunological improvement at the time of surgery could be explained by the massive release of tumour antigens that could determine an exhaustion of the immunological response [31].

In rOC patients, the behaviour of T-cell subsets was similar to what observed in the control group. This unexpected phenomenon could support the immunoediting theory of tumour progression [24]. It is possible that cancer clones, which survived primary treatments, are selected to be poorly immunogenic as supported by the observation that most patients were affected by nodal recurrences. Another hypothesis is that the immune system may be too aberrant by the time of second surgery, and that signalling and cell function may be sufficiently compromised that surgery is not able to restore functionality.

The prognostic significance of circulating cells goes beyond our study objectives. However, we observed a significant increase

in circulating CD8/CD4 T_{reg} ratio in the group of patients treated with the gold standard treatment and therefore the best expected prognosis. Considering the above information, our data show that primary cytoreduction has an important immune activating effect. In addition our results suggest that future studies, that directly address the relationship between patients' immunological status and prognosis, should be carried out.

Primary tumour debulking is effective in reducing T_{reg} by acting on the primary cause and therefore reduces replenishment by conversion [32, 33]. This capacity of surgery is lost or reduced when surgery is not carried out as first treatment. It should be noted though, that patients in the pOC group, were the ones with the greatest tumour burden before surgery and smaller variations in the immune system of IDS and rOC patients might require much greater numbers to be highlighted. Our observations suggest that the best strategy to be adopted for managing T_{reg} cells during immunotherapy trials is different depending on the setting in which it is being applied. The results obtained in our study suggest that patients subjected to primary cytoreduction or respond to NACT, and in whom visible tumour bulk was completely removed, represent ideal candidates for testing novel cancer vaccination protocols.

In conclusion, this study demonstrates that surgery is able to partially revert the immunosuppression state of cancer patients and re-establishing a physiological immunological balance. Surgery plays its greatest immunological effects when it is carried out as primary treatment. These results will aid the scientific community to develop new therapeutic strategies and develop more efficient immunotherapy vaccination schedules.

Acknowledgements

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