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Foxp3$^+$ cell infiltration and granzyme B$^+$/Foxp3$^+$ cell ratio are associated with outcome in neoadjuvant chemotherapy-treated ovarian carcinoma

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Abstract Preoperative neoadjuvant chemotherapy (NAC) can significantly reduce tumour burden in patients with primarily unresectable chemosensitive tumours, allowing a more complete cytoreduction during debulking surgery and facilitating evaluation of tumour chemosensitivity, identification of appropriate treatment options and improvement of intervention protocols. In this study, we investigate, using immunohistochemistry, the impact of platinum/taxane-based NAC (NAC) on tumour-infiltrating lymphocytes (TILs) in advanced epithelial ovarian cancer (EOC) and their relationship with clinical outcome. All patients had clinical response, as shown by ascites volume and CA125 levels compared to pre-treatment findings. NAC intervention significantly increased CD4$^+$, CD8$^+$ and granzyme B$^+$ infiltration while Foxp3$^+$ accumulation remained unaffected. TILs were prognostically neutral for both progression-free survival (PFS) and overall survival (OS) before NAC. In contrast, after NAC, elevated granzyme B$^+$ infiltration displayed a tendency for improved PFS (log-rank 0.064). Further, low Foxp3$^+$ cell density was associated with longer PFS, as compared with strong Foxp3$^+$ infiltration (median 20.94 vs. 11.24 months; log-rank 0.0001) and with improved OS (median 30.75 vs. 16.04 months, respectively; log-rank 0.056), demonstrating clear prognostic significance for PFS. In addition, high granzyme B$^+$/Foxp3$^+$ ratio post-NAC strongly correlated with improved PFS compared to low granzyme B$^+$/Foxp3$^+$ cell ratio (median 17.88 vs. 11.24 months, respectively), and showed to be a favourable prognostic factor for PFS (log-rank 0.014). Our findings indicate that NAC elicited an immunologic profile in which low immunosuppressive Foxp3$^+$ infiltration and elevated numbers of activated granzyme B$^+$ cells were significantly associated with EOC-specific PFS, suggesting a contribution of immunologic effects to improved clinical outcome.

Keywords Neoadjuvant chemotherapy · Ovarian cancer · TILs · CD8/CD4 ratio · Foxp3 · Granzyme B

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Introduction

In industrialized countries, epithelial ovarian cancer (EOC) is the main cause of death in women with gynaecologic malignancies. Its bleak outcome is due to the fact that more than 70% of patients present with advanced-stage disease FIGO III/IV (International Federation of Gynaecology and

Obstetrics). Although 70% of them respond well to standard management, primary radical tumour debulking followed by platinum/taxane-based chemotherapy, the majority of patients relapse and die of progressive disease [1]. Patients with FIGO stage IIIC and IV disease have an unfavourable prognosis with 5-year survival rates varying from 19 to 33% depending on resectability and response to chemotherapy [2], the extent of cytoreductive surgery and the amount of postoperative residual tumour being among the most significant prognostic factors impacting on survival [3]. However, complete tumour resection cannot be achieved in a substantial number of EOC patients. In patients with primarily unresectable chemosensitive tumours, neoadjuvant chemotherapy (NAC)—i.e. primary chemotherapy followed by tumour debulking—can considerably reduce tumour load, allowing a higher degree of cytoreduction by interval laparotomy [4–7]. In addition, NAC enables, by measuring residual disease in surgical specimens assessment of tumour chemosensitivity, definition of appropriate treatment options and improvement of intervention protocols.

Over the years, administration of systemic chemotherapy for the treatment of human malignancies has been believed to exert deleterious effects on the host's defense system, particularly in advanced-stage patients who may as well be immune compromised. However, more recent data suggest that certain cytotoxic drugs can promote antitumour immune responses that contribute to the therapeutic effects of conventional therapy [8–10]. In this respect, recent work has provided evidence for synergistic immuno-adjuvant effects of taxane-based chemotherapy combined with tumour vaccines in experimental models and cancer patients [10]. It is by now a well-established notion that lymphocyte infiltration is a crucial regulation mechanism in oncogenesis and an indication of the host antitumour response [11, 12]. Although histopathologic alterations following NAC treatment of advanced-stage EOC have already been reported [13], and the prognostic significance of T cell infiltration has lately received considerable attention [14–20], the effect of NAC on tumour/host interactions in situ has, despite the increasing appreciation of this regime in the management of EOC, not been studied so far. Based on these premises, we conjectured that NAC-induced cytotoxicity may impact—by exerting local tissue disruption and/or release of pro-inflammatory factors by tumour cells themselves or by recruited immune cells—upon influx, phenotype or functional state of T cell infiltrates, and that these alterations may affect disease outcome and can thus be exploited therapeutically.

Therefore, we aimed at measuring immunohistochemically the extent of tumour-infiltrating $CD4^+$, $CD8^+$, granzyme B^+ and $Foxp3^+$ T cells as well as the ratio of $CD8^+$/ $CD4^+$, $CD4^+$/$Foxp3^+$, $CD8^+$/$Foxp3^+$ and granzyme B^+/ $Foxp3^+$ T cells in archival formalin-fixed paraffin-embedded biopsy specimens of 30 EOC patients prior to and after NAC treatment. Immune infiltrates were further analysed with regard to clinicopathologic variables known to impact on disease prognosis, including histological grade and presence of residual disease. This study is, to our knowledge, the first to demonstrate significant association between Foxp3 and granzyme B expression levels with clinical outcome in NAC-treated EOC patients. Our findings are particularly relevant in view of recent reports indicating that chemotherapeutic intervention and/or depletion or functional inactivation of $Foxp3^+$ regulatory T cells (Tregs) may significantly enhance the efficacy of cancer vaccination protocols [9, 21].

Materials and methods

Patients and clinical parameters

From March 2003 to November 2007, a total of 93 patients with histologically confirmed EOC at FIGO IIIC and IV and ascites volume ≥500 mL were included into a prospective multicenter phase II study (Primovar) [7]. Trial objectives were evaluation of response to NAC and analysis of surgical outcome. Treatment consisted of either two or three of six cycles of i.v. administered carboplatin (AUC 5) and docetaxel (75 mg/m^2) prior to cytoreductive surgery. Cytoreductive surgery was done within 4 weeks after the last scheduled chemotherapy cycle. All patients were regularly followed at 3-month intervals for the first 2 years and at 6-month intervals thereafter. The study received approval from the local institutional review board and all patients gave written informed consent.

The analysis of lymphocytic tumour infiltration and clinical follow-up was done on samples obtained from 30 out of 35 patients enrolled at our institution having adequate formalin-fixed, paraffin-embedded tumour specimens for immunohistochemistry, as well as complete clinicopathologic data. Intraperitoneal biopsies were collected by laparoscopy or laparotomy prior to chemotherapy and at debulking laparotomy at the Department of Gynaecology and Obstetrics of the Bonn University Medical Centre.

Immunohistochemistry of tissue specimens

Tissue samples were fixed in formalin and embedded in paraffin by standard procedures. For immunostaining, 2-μm sections mounted on polylysine-coated slides were deparaffinized, rehydrated, subjected to a heat-induced epitope retrieval step in a high-pressure cooker (30 min in

10 mM sodium citrate buffer, pH 6.0) and rinsed in Tris-buffered saline pH 7.4 for 20 min at room temperature. Endogenous peroxidase activity was blocked by 7.5-min incubation in Dako REAL™ Peroxidase Blocking Solution. Immunostaining was performed by incubating the slides at 4°C over night with mouse monoclonal antibodies against CD4 (clone 1F6, 1:120; Novocastra, Newcastle Upon Tyne, UK), CD8 (clone C8/144B, 1:25; DakoCytomation, Hamburg, Germany), Foxp3 (clone 236A/E7, 1:50; ebioscience, San Diego, USA) and granzyme B (clone 11F1, 1:20, Axxora, Lörrach, Germany). Labelling was detected using the Dako REAL™ Detection System, Streptavidin Peroxidase/AEC according to the manufacturer's instructions. For Foxp3, tonsillar tissue with follicular hyperplasia showing T cells in the interfollicular area with nuclear antigen expression served as positive control. Negative controls were done by omitting the primary antibody. The stained sections were then counterstained with hematoxylin, rinsed with water and mounted with glycerol gelatine.

Quantitative assessment of tumour-infiltrating lymphocytes (TILs)

Stained TILs were assessed on representative, independent high power magnification-scanned (400×) areas of serial sections with the highest TIL density. These zones were digitally photographed, and to minimize the heterogeneous distribution of positive cells, a minimum of four high power fields (1 hpf = 0.237 mm^2) for each patient sample was selected, counted manually at least three times by two different observers blinded for the patients' clinical data (EB, NF) and the averaged counts in each case was statistically evaluated. Only viable tissue was included in the area of analysis, and only stained cell infiltrates in tumour cell nests—but not in the stromal compartment—were considered for evaluation. Median values were used as cut-offs to define subgroups with high or low infiltration for all immunohistochemical markers.

Statistical analysis

Statistical analyses were performed with the SPSS and SAS software programs. Cumulative survival time was estimated using the Kaplan–Meier method and the survival difference was detected by the two-sided log-rank test for univariate analysis. The level of significance was set at $P < 0.05$. Data were censored at the last follow-up for patients who were disease-free or alive at the time of analysis. Comparisons of TIL counts in pre- and post-chemotherapeutic specimens were carried out using the t test and $P < 0.05$ was considered significant.

Results

Clinical patient profile

The clinical and histological characteristics of the patients included in this study are summarized in Table 1. The median age of the study population at the time of diagnosis was 62 years (range 39–80). Of 30 patients, 29 were diagnosed as FIGO IIIC and 1 as FIGO IV. Histological subtypes of the tumour comprised 29 cases of serous/serous-papillary and 1 case of endometrioid carcinoma. All patients were classified as responders to initial chemotherapy, as shown by ascites volume and CA 125 levels compared to pre-treatment findings. All patients received a total of six cycles of chemotherapy. Towards the end of the

Table 1 Patient characteristics

	Total
Number of patients	30
Age (years)	
Median	62
Range	39–80
PFS (months)	
Median	13.3
Range	3.8–50.7
OS (months)	
Median	33.1
Range	3.8–65
CA 125 (U/ml)	
Median	1,412
Range	43–9,030
Histological type (no.)	
Serous/serous-papillary	29
Endometrioid	1
Histological grade (no.)	
G2	12
G3	18
Stage (no.)	
III C	29
IV	1
Number of chemotherapy cycles before cytoreductive surgery (no.)	
2	17
3	13
Residual tumour (no.)	
>1 cm	4
≤1 cm	17
No macroscopic tumour	9
Mortality and recurrence status (no.)	
Recurrent disease	22
Dead	16
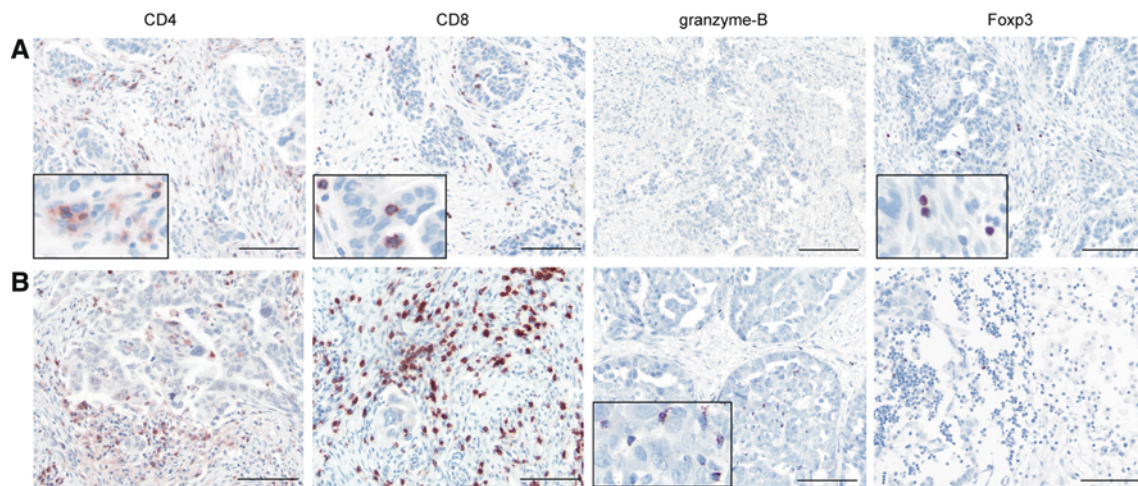
Fig. 1 Immunohistochemical staining pattern of intratumoral CD4+, CD8+, granzyme B+ and Foxp3+ T cells in representative specimens of EOC patients (serous type, FIGO IIIc, grade G3, ×400 magnification) prior to (**a**) and after preoperative neoadjuvant chemotherapy (**b**). *Scale bar* 100 μm

study, 22 (73%) patients had developed recurrent disease, 16 (53%) succumbed to their disease, and 14 (47%) were still alive. The mean follow-up period was 24 months (range 7–65). The median CA 125 levels pre- and post-NAC treatment were 1,412 U/ml (range 43–9,030) and 59 U/ml (range 8–1,872), respectively. The median progression-free (PFS) and overall survival (OS) for all patients examined were 13.3 and 33.1 months (range 3.8–50.7 and 3.8–65 months), respectively.

Analysis of T cell infiltration before and after NAC intervention

Antitumour immune response to chemotherapy was analysed by immunohistochemical staining of intratumoral CD4+, CD8+, granzyme B+ and Foxp3+ T cells in ovarian epithelium surgical specimens as well as by assessing the ratio of CD8+/CD4+, CD4+/Foxp3+, CD8+/Foxp3+ and granzyme B+/Foxp3+ T cells prior to and after NAC. Immune cells infiltrated tumour tissue in a disseminated manner as scattered solitary cells, as shown by hematoxylin staining, and displayed low level of homogeneity and broad inter-individual differences for stained cell density, the number of CD4+, CD8+, granzyme B+ and Foxp3+ cells varying significantly among samples. While CD4 and CD8 displayed cell membrane staining and were vigorously expressed, a weak accumulation of granzyme B+ and Foxp3+ cell infiltrates was observed (Fig. 1). Foxp3 showed, in line with its function as a transcription factor distinct nuclear localization. Granzyme B, a granule-associated protein crucial for cytolytic function, is constitutively expressed by NK cells and by activated but not by naïve CD8+ CTLs [22]. Here, we identified only cells with sparsely granulated cytoplasmic pattern as stimulated cytotoxic T lymphocytes (CTLs), while densely positive natural killer (NK) cells were disregarded. Several reports have demonstrated the prognostic significance of intraepithelial—in contrast to stromal—CD8+ T cell infiltration in patients with ovarian [15, 18–20], endometrial [23], cervical [24], colorectal [25] and hepatocellular cancer [26]. In this report, an explicit analysis of stromal-infiltrating lymphocytes was not performed, as the stromal component might be caused not just by the tumour itself (epithelial-to-mesenchymal transition, EMT) but also by scar tissue following therapy-induced necrosis. This NAC effect may, therefore, not be properly distinguished from tumour-induced EMT, so that stromal lymphocytes in this neoadjuvant setting might reflect not only an immune response to tumour stroma but also an unspecific immune response within scar tissue. Accordingly, we assessed here only lymphocyte infiltrates in the tumour epithelium itself and those immediately contacting it. Representative images of lymphocyte infiltrates showing antigen expression levels prior to and after NAC as well as descriptive statistics of immunohistochemical variables are shown in Fig. 1 and Table 2, respectively.

The mean number of infiltrating CD4+, CD8+ and granzyme B+ cells was significantly enhanced after NAC, as compared to cell accumulation prior to NAC (CD4: 2.5-fold increase, CD8: 2.5-fold; granzyme B: 2.5-fold) whereas Foxp3+ cell numbers were equivalent in pre- and post-NAC samples (Fig. 2a). These findings argue for a specific differential effect of NAC on recruitment or expansion of conventional and activated T cells on one side and Tregs on the other side. In addition, CD8+/Foxp3+ and granzyme B+/Foxp3+ cell ratios were significantly elevated after NAC (2.6-fold and 2.4-fold increase, respectively), whereas CD8+/CD4+ remained unaffected by chemotherapy (Fig. 2b).

Table 2 Descriptive statistics of tumour infiltrating lymphocytes

	$CD4_A$	$CD4_P$	$CD8_A$	$CD8_P$	GrB_A	GrB_P	$Foxp3_A$	$Foxp3_P$	$CD8_A/CD4_A$	$CD8_P/CD4_P$	$CD4_A/Foxp3_A$	$CD4_P/Foxp3_P$	$CD8_A/Foxp3_A$	$CD8_P/Foxp3_P$	$GrB_A/Foxp3_A$	$GrB_P/Foxp3_P$
Range[a]	0–25.5	0–75	0–51	6.5–139	0–8.5	0–18	0–19.5	0–11	0–13	0.34–5	0.77–20.0	0–57.0	0.66–36	2–101	0–2.5	0–9
Mean[a]	8.35	20.95	14.46	37.01	1.98	4.96	3.55	3.61	1.86	2.33	3.34	9.35	7.29	19.14	0.75	1.82
Std Dev	7.53	19.63	12.39	27.89	2.31	4.08	4.85	3.29	2.49	1.35	4.45	12.7	9.72	24.95	0.87	2.22
Median[a]	8.00	14.25	11.25	26.25	1.5	4.50	1.5	3.75	1.38	1.96	2.33	3.11	5.5	7.57	0.3	1.15
Cases (n)	30	30	30	30	30	30	30	30	24[b]	29[b]	19[b]	25[b]	19[b]	25[b]	19[b]	25[b]

A ante-NAC, P post-NAC, GrB granzyme B

[a] Number of TILs per high power field before (A) and after (P) preoperative neoadjuvant chemotherapy (NAC)

[b] The discrepancy in the case numbers is due to the division by zero in a quotient. The denominator (FOXP3+ cells) in the corresponding samples was zero, meaning that we did not observe FOXP3+ infiltrating cells in those specimens
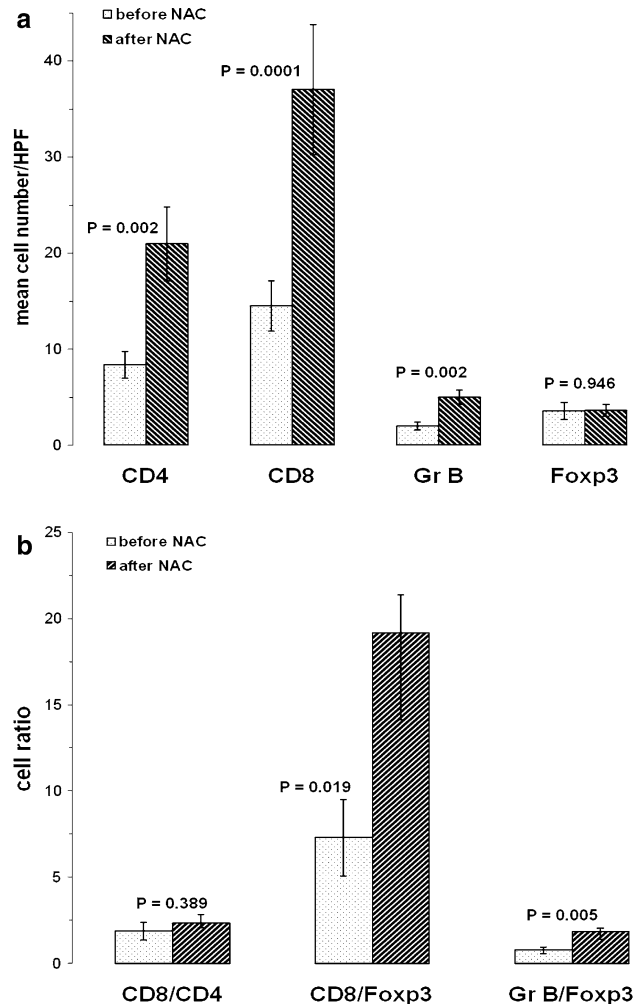
Fig. 2 Comparison of immune cell infiltrates in specimens of EOC patients prior to and after neoadjuvant chemotherapy. *Columns* represent the mean number of cells per high power field. Statistical analysis was done using the *t* test

Correlation of clinicopathologic and immunohistochemical variables with clinical outcome

On Kaplan–Meier analysis, clinical variables such as histological grade or number of preoperatively administered chemotherapeutic cycles did not show any prognostic significance for either PFS or OS (Table 3). In patients with no macroscopic evidence of residual disease after cytoreductive surgery, which has been considered the most significant predictor of EOC survival [3], longer OS than in those with tumour load was demonstrated, although without attaining full statistical significance (median 65.06 vs. 33.17 months, respectively; log-rank 0.075). To assess the influence of high versus low levels of infiltrating cells on disease prognosis, we used the corresponding median as cut-off for each lymphocyte population (Tables 2, 3). None of the T cell

Table 3 Univariate analysis of clinicohistochemical variables and survival in neoadjuvant chemotherapy-treated EOC patients

Variables	PFS			OS		
	Median[a]	95% CI[a]	Log-rank	Median[a]	95% CI[a]	Log-rank
Histological grade						
G2 = 12	13.31	6.25–15.22	0.164	33.17	10.19–65.06	0.617
G3 = 18	13.28	11.05–32.91		35.96	15.45–62.10	
NAC cycles						
2 = 17	14.53	10.36–24.00	0.984	33.17	16.04–n.e.	0.860
3 = 13	13.28	6.25–32.91		35.96	10.19–65.06	
Residual tumour status						
Yes = 21	12.62	10.36–14.53	0.189	33.17	14.83–36.75	0.075
No = 9	15.22	8.61–n.e.		65.06	16.04–65.06	
$CD4_A$						
>8.0	14.53	10.85–n.e.	0.474	36.75	14.60–n.e.	0.660
<8.0	13.28	8.61–15.22		20.87	14.83–62.10	
$CD4_P$						
>14.25	12.62	8.61–13.87	0.192	20.74	14.66–36.75	0.133
<14.25	17.88	10.36–n.e.		35.96	14.83–65.06	
$CD8_A$						
>11.25	13.28	8.61–n.e.	0.859	20.74	14.66–62.09	0.831
<11.25	13.87	10.36–17.88		33.17	15.02–65.06	
$CD8_P$						
>26.25	12.62	8.61–15.22	0.547	20.87	16.04–65.06	0.897
<26.25	13.58	10.36–32.91		35.96	14.83–n.e.	
GrB_A						
>1.5	15.22	10.36–n.e.	0.217	65.06	14.63–65.06	0.089
<1.5	12.62	8.61–17.88		20.87	15.45–36.75	
GrB_P						
>4.5	14.53	10.85–n.e.	0.064	–	–	0.474
<4.5	11.87	8.32–15.22		33.17	14.83–62.10	
$Foxp3_A$						
>1.5	12.62	6.67–15.22	0.233	33.17	14.45–65.06	0.743
<1.5	14.53	11.24–32.91		35.91	14.83–62.10	
$Foxp3_P$						
>3.75	11.24	8.32–12.75	0.0001	16.04	14.63–33.07	0.056
<3.75	20.94	11.87–n.e.		30.75	15.02–65.05	
$CD8_A/CD4_A$						
>1.38	11.41	8.32–32.91	0.742	33.17	14.63–62.10	0.650
<1.38	13.28	11.05–24.00		36.75	15.02–n.e.	
$CD8_P/CD4_P$						
>1.96	15.22	8.32–n.e.	0.206	62.10	16.04–62.06	0.032
<1.96	12.75	10.36–14.53		20.74	14.83–35.96	
$CD4_A/Foxp3_A$						
>5	14.53	10.36–26.17	0.741	20.87	14.66–36.75	0.620
<5	12.75	8.32–15.22		33.17	14.63–65.06	
$CD4_P/Foxp3_P$						
>1.15	14.53	10.36–26.17	0.620	35.96	14.83–65.06	0.734
<1.15	13.28	8.32–24.00		33.17	14.63–62.10	

Table 3 continued

Variables	PFS			OS		
	Median[a]	95% CI[a]	Log-rank	Median[a]	95% CI[a]	Log-rank
$CD8_A/Foxp3_A$						
>5.5	14.53	10.36–32.91	0.835	20.87	14.66–62.10	0.415
<5.5	13.28	8.32–24.00		33.17	15.45–35.96	
$CD8_P/Foxp3_P$						
>7.57	15.22	10.36–32.91	0.119	35.96	14.83–65.06	0.528
<7.57	12.08	8.32–14.53		16.50	14.63–62.10	
$GrB_A/Foxp3_A$						
>0.30	17.88	11.24–n.e.	0.102	36.75	14.66–65.06	0.215
<0.30	10.85	6.25–13.28		20.74	10.19–33.16	
$GrB_P/Foxp3_P$						
>1.15	17.88	10.85–32.91	0.014	35.96	16.04–65.06	0.137
<1.15	11.24	6.67–13.28		33.17	14.63–62.10	

PFS progression-free survival, *OS* overall survival, *NAC* neoadjuvant chemotherapy, *A* ante-NAC, *P* post-NAC, *GrB* granzyme B, *n.e.* not estimable

[a] Values in months

infiltrates examined were associated with improved clinical outcome in pre-chemotherapeutic specimens. In contrast, after NAC, patients with high granzyme B cell infiltration (>4.5) showed, as compared to those with low cell density (<4.5), a tendency towards an extended PFS (median 14.53 vs. 11.87 months, respectively; log-rank 0.064), whereas no association was found with OS (Table 3). In addition, the group of patients with low intratumoral Treg density after NAC (<3.75) exhibited longer PFS and OS (median 20.94 and 30.75 months, respectively) than did that with prevalent Foxp3$^+$ (>3.75) cell infiltration after NAC (median 11.24 and 16.04 months, respectively), and disclosed significant association with improved PFS (log-rank 0.0001 and 0.056 for PFS and OS, respectively, Table 3). The development of Foxp3$^+$ cell infiltrates in all EOC patients prior to and after NAC intervention is illustrated in Fig. 3. However, none of these factors reached significant independent prognostic influence in a multivariate analysis (data not shown).

Accruing reports have recently portended that the specific type, functional state, balance and distribution of infiltrating immune cells rather than their absolute numbers critically determine their prognostic significance [15, 26, 27]. Accordingly, to examine the role of T cell subsets with ancillary or antagonistic functions, we assessed the impact of CD8$^+$/CD4$^+$, CD4$^+$/Foxp3$^+$, CD8$^+$/Foxp3$^+$ and granzyme B$^+$/Foxp3$^+$ cell ratio on patient survival, using as aforementioned median value as cut-off (Tables 2, 3). High intratumoral CD8$^+$/Foxp3$^+$ cell ratio, which has been consistently reported as a significant predictor of clinical outcome in ovarian [15], cervix [24], breast [28] and colorectal carcinoma [29], was not associated with improved survival of EOC patients, neither prior to nor after
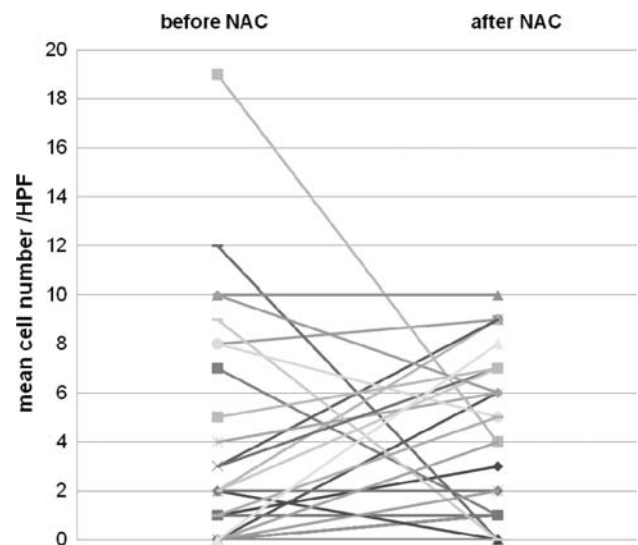
Fig. 3 Development of Foxp3$^+$ cell infiltrates in EOC prior to and after NAC intervention

chemotherapy (Table 3). In addition, whereas CD8$^+$/CD4$^+$ T cell ratio prior to NAC had no influence on either PFS or OS, high CD8$^+$/CD4$^+$ T cell ratio after NAC (>1.96) was predictive of improved OS (median 62.10 vs. 20.74 months, respectively; log-rank 0.032) (Table 3). Significantly, and reinforcing these findings, high granzyme B$^+$/Foxp3$^+$ cell density after NAC (>1.15) correlated significantly with extended PFS, as compared to that of patients with a low (<1.15) granzyme B$^+$/Foxp3$^+$ cell ratio (median 17.88 vs. 11.24 months, respectively), and proved clearly as a prognostic parameter (log-rank 0.014, Table 3).

Taken together, these data indicate that preoperative NAC specifically facilitated increased intratumoral

accumulation of CD4$^+$, CD8$^+$ and effector granzyme B$^+$ cells, whereas Foxp3$^+$ cell infiltration remained unaffected. In addition, while Foxp3 infiltration prior to NAC treatment was not predictive of survival, weak Foxp3$^+$ cell influx following NAC was associated with prolonged OS and PFS and conversely, a higher Treg recruitment post-NAC was a significant poor predictor of EOC. Accordingly, low numbers of FOXP3$^+$ cells in combination with enhanced infiltration of activated granzyme B$^+$ CTLs after NAC proved to be a positive prognostic factor associated with increased PFS but not with improved OS.

Discussion

A large body of evidence has documented that high T cell infiltration augurs a favourable outcome in a variety of human cancers [11, 12]. Endorsing this paradigm, an early study of EOC showed that the presence of intratumoral T cells correlated independently with improved PFS and OS, and was associated with enhanced expression of IFN-γ, IL-2 and lymphocyte-attracting chemokines within the tumour, suggesting activation of antitumour immunity [14]. Ensuing data demonstrated that active accumulation of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells in EOC was associated with suppression of tumour-specific cytotoxicity and cytokine production by CD8$^+$ T cells, growth of human tumours in vivo, high death hazard and reduced patient survival [17]. In line with these findings, Foxp3 mRNA expression in EOC was shown to be an independent adverse prognostic factor for PFS and OS [16], indicating that high Foxp3 expression levels may represent a surrogate marker for an immunosuppressive milieu fostering tumour immune escape. Tregs—either natural or inducible—mediate peripheral self-tolerance and preclude protective antitumour immunity by suppressing effector T cell proliferation and cytokine production through soluble- and/or contact-dependent factors [30, 31]. Foxp3, a member of the forkhead/winged helix family of DNA transcription factors, is essential for Treg development and function and represents, thus far, the most selective Treg marker [32]. Yet, more comprehensive analyses have recently indicated that the specific T cell subsets and their mutual interactions rather than the absolute numbers control the host-versus-tumour reaction and predict disease outcome [15, 26, 27]. Accordingly, only intraepithelial CD8$^+$ T cell recruitment and high CD8$^+$/CD4$^+$ and CD8$^+$/CD25$^+$FoxP3$^+$ ratio were associated with favourable prognosis in EOC, Foxp3$^+$ cells attenuating the protective effects of CD8$^+$ effector cells [15].

In this report, we show that platinum/taxane-based neoadjuvant intervention significantly increased the frequencies of CD4$^+$, CD8$^+$ and granzyme B$^+$ cells in ovarian surgical specimens as compared to NAC naïve samples, whereas Foxp3$^+$ accumulation remained unaffected. Furthermore, while the CD8$^+$/CD4$^+$ cell ratio was not influenced by chemotherapy, CD8$^+$/Foxp3$^+$ and granzyme B$^+$/Foxp3$^+$ were notably enhanced after NAC. These findings suggest either differential expansion or recruitment of conventional T cells and Tregs as a result of chemotherapy, arguing for a specific treatment-associated antitumour immune response. Indeed, in accordance with this notion, previous reports have demonstrated that cytotoxic agents may modulate T cell infiltration, improving thereby clinical outcome. In this respect, a study of cervical carcinoma patients pre-surgically treated with chemotherapy or chemoradiation revealed increased incidence of activated T cells and a concomitant reduction of naïve T cells in uninvolved lymph nodes (LN), whereas the high levels and suppressive function of CD4$^+$CD25^{high}CD152$^+$ Tregs were unaffected. Of note, LNs from patients with an objective response contained significantly higher numbers of activated T and NK cells and diminished frequencies of Tregs, indicating an enhanced treatment-induced capacity of the local immune response to mediate antitumour effects [33]. In addition, a study of B cell chronic lymphocytic leukaemia (CLL) demonstrated significant stage-dependent increase of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in peripheral blood of individuals with extended disease, but reduced frequencies and decreased or abrogated Treg inhibitory function after therapy with fludarabine [34]. Most importantly, carboplatin/paclitaxel-based chemotherapy of EOC patients displaying high CA 125 levels and compromised T cell function was shown to affect significantly decreased CA 125 levels and restore potent CD8$^+$ T cell responses in patients at remission, pointing at an association between chemotherapy, T cell function and improved clinical outcome [35].

Several studies have identified prevalence of CD8$^+$ T cells and high CD8$^+$/CD4$^+$ or CD8$^+$/Treg ratio as significant predictors of survival in EOC [15, 18, 20], cervical [24], breast [28], colorectal [25] and hepatocellular cancer [26]. In contrast, in the current report, only an elevated CD8$^+$/CD4$^+$ cell ratio was found to have a positive prognostic influence on OS on Kaplan–Meier analyses, whereas neither CD8$^+$ cell infiltration nor CD8$^+$/Treg ratio was predictive of outcome. Consistent with our findings, no association of CD8$^+$ TILs with improved prognosis has been documented in some other malignancies including lung cancer [36], anal squamous cell and hepatocellular carcinoma [26, 37], indicating that the effects of CD8$^+$ cell infiltration may vary in different tumour types. Further aspects contributing to these conflicting results may include use of different detecting antibodies, preceding treatment modalities, as well as disease stage and immune status of the patients under study.

The most significant results of this study are the correlation of enhanced granzyme B$^+$ cell density following NAC with improved PFS as well as the finding that

treatment-associated decreased Foxp3$^+$ cell infiltration is a statistically significant favourable prognostic factor for PFS, whereas patients with higher Treg numbers reveal poorer survival. In accordance with our results, abundance of Foxp3$^+$ infiltrates has been linked to adverse outcome in various types of solid tumours including EOC [16, 17], endometrial [38], breast [39, 40], pancreatic [41] and hepatocellular cancer [42, 43]. By contrast, low Foxp3$^+$ cell density has been shown to represent an independent prognostic factor negatively influencing survival in Hodgkin's lymphoma [44], and high Treg numbers predicted improved survival in follicular lymphoma [45] and colorectal cancer [46], suggesting that, analogous to CD8$^+$ cells, Tregs may not play equivalent roles in different neoplasms. Our findings are further reinforced by the observation that increased influx of activated granzyme B$^+$ T cells counteracting the adverse prognostic effects of Treg infiltration is a significant predictor of decreased risk of recurrence, arguing for local NAC-induced immune surveillance. Indeed, recent data strongly indicate that chemotherapy-induced accumulation of apoptotic cells may foster tumour immunogenicity by providing tumour antigens for cross-presentation by dendritic cells (DC) to T lymphocytes [8]. Remarkably, one study of advanced breast cancer showed that neoadjuvant paclitaxel chemotherapy induced intratumoral apoptotic response that correlated with TIL recruitment in 67% of patients developing complete clinical response with pathological residual disease [47].

Our data indicate further that not the extent of CD8$^+$ cell infiltration, but the activation state is critical for its prognostic significance. In this respect, the presence of high intratumoral granzyme B$^+$ effector CTLs and low Treg levels was shown to be an independent prognostic factor for improved outcome in hepatocellular carcinoma [26]. Most importantly, pathological complete response to a preoperative anthracycline/taxane-based NAC in patients with breast carcinoma has been associated with an immunologic profile combining high levels of infiltrating CD8$^+$ and cytotoxic granzyme B$^+$ cells and absent or decreased Foxp3$^+$ cell infiltrates [28].

In summary, the current study is, to our knowledge, the first to analyse the impact of carboplatin/taxane-based NAC on the lymphocyte composition of surgical specimens of patients with EOC. Systemic chemotherapy for the management of cancer has previously been perceived to exert deleterious effects on the host's defense system, particularly in advanced-stage patients who may as well be immune compromised. Accordingly, patients undergoing cytotoxic treatment within 2 months before study entry have been excluded thus far from most immunotherapeutic trials. Although the limited number of patients studied does not allow definitive conclusions, our results underscore Foxp3$^+$ cell infiltration as adverse prognostic factor and

present evidence of a treatment-dependent increase of activated granzyme B$^+$ T cells exerting a beneficial effect on disease outcome. Although the mechanisms underlying such an effect have not elucidated here, we conjecture that chemotherapy-induced apoptosis may induce, via cross-presentation of tumour antigens by DCs, increased levels of primed granzyme B$^+$ effector cells which might hamper tumour progression and contribute to improved survival. Recent studies have reported that Treg depletion substantially enhances vaccine-mediated antigen-specific effector T cell immunity resulting in occasional therapeutic responses in several human malignancies [48, 49]. Based on the findings presented here, as well as previous data from our laboratory demonstrating the efficacy of a DC-based immunotherapy for relapsed metastatic EOC [50], we propose that administration of platinum/taxane-based NAC prior to tumour vaccination may, by inactivation or depletion of infiltrating Foxp3$^+$ cells in association with increased accumulation of activated granzyme B$^+$ cells, stimulate antitumour immune responses impacting favourably on clinical outcome of EOC patients.

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