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Accumulation of CD45RO⁺ cells in peritoneal carcinomatous fluid favours survival of ovarian carcinoma patients

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Abstract In 44 patients with advanced ovarian carcinoma (OC) a fraction of CD45RO⁺ lymphocytes in the blood and peritoneal carcinomatous fluid (PCF) was investigated. Thirty-one patients received cisplatin with cyclophosphamide ± doxorubicin. This group was followed from 2.2 to 9 years (mean: 45 months). In 23 out of 31 patients, the percentage of CD45RO⁺ lymphocytes was higher in the PCF than in the blood samples. Patients with these higher lymphocyte levels experienced longer survival than those who did not show any excess of CD45RO⁺ lymphocytes in PCF ($P=0.02$). This was further verified by the use multivariate Cox analysis which included an assessment of the percentage of CD45RO⁺ lymphocytes in PCF, age, FIGO status, histology, treatment (CAP or CP) and residual disease (RD) post-surgery. This analysis revealed that two factors had an independent power of prediction: RD ($P=0.02$) and the percentage of CD45RO⁺ cells in PCF ($P=0.04$). Therefore, CD45RO⁺ lymphocytes were studied in further detail in a group of 20 patients. This study revealed that PCF CD45RO⁺ lymphocytes were characterized by: (1) a higher proportion of cells co-expressing activation markers (HLA-DR, CD28) and higher levels of mRNA for CXC chemokines (IP-10, IL-8) and for IL-10, but with lower levels for IL-2; (2) higher levels of Ki67, bcl-2 and p53 mRNA as compared to those in blood. In conclusion, in the present study it

was found that an accumulation of activated CD45RO⁺ cells in PCF had a beneficial effect on the survival of patients receiving platinum-based chemotherapy.

Keywords CD45RO · Chemokine · Ovarian carcinoma · Survival

Introduction

In patients with advanced ovarian carcinoma (OC), the rate of survival is known to be low [13]. This is due to (1) emerging chemoresistant clones; and (2) colonisation of the peritoneum, resulting in poor accessibility by chemotherapeutic agents [22]. However, peritoneal metastatic lesions when at an early stage and microscopic in size, may be attacked by agents involved in immunological surveillance. OC tissue generates several cytokines that may affect both tumour (in an autocrine manner) and tumour-infiltrating cells [18]. OC cells are CD95⁺ and this renders them susceptible to CD95 ligand-mediated apoptosis [1, 2]. These characteristics provide a rationale for immunotherapeutic investigation. Several protocols have included the use of either IL-2 [5] or IFN-gamma [21] as adjuvants to chemotherapy. Anti-FAS (CD95) monoclonal antibodies are used for neoplastic cell eradication [26] Hoskins et al. assumed from survival analysis of early relapsing OC cases that surveillance of this disease plays an important role in its natural history [8]. In this study, we report that an excess of CD45RO⁺ cells in peritoneal carcinomatous fluid (PCF) favours survival even in patients with more advanced (FIGO > IIC) OC.

Materials and methods

Patients

The present study included 44 OC patients. Thirty-three patients were allocated randomly for PAC (6 courses of cisplatin 50 mg/m², doxorubicin 50 mg/m² and cyclophosphamide 500 mg/m² every 3 weeks) or for PC (7 courses of cisplatin 75 mg/m² and

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cyclophosphamide 750 mg/m² every 3 weeks) before 1998. Two of these patients died before receiving 4 cycles of treatment, thus their data were excluded from the survival analysis. From 1998 onward, all patients received paclitaxel combined with a cisplatin-based regimen. Patients received high-dose chemotherapy with stem cell support to consolidate routine chemotherapy. Patients on paclitaxel alone and those who received high-dose or intraperitoneal chemotherapy were excluded from the survival analysis (Table 1).

Peripheral blood mononuclear cells and PCF cell purification

PCF volume ranged from 0.25 to 20 l. Cancer and mesothelial cells tended to form aggregates what made it difficult for them to be counted. Only in 17 patients was enumeration of PCF possible. In these cases, the PCF cell count ranged from 0.042 to 9.6×10⁶ cells/ml, with a median of 2.4×10⁶ cells/ml ± 2.1 SEM. The same was the case for leukocytes and mesothelial cells, whose number varied from 1.7×10⁶ cells/ml ± 1.5 SEM to 1.0×10⁶ cells/ml ± 1.1 SEM, respectively. All harvested PCF, if the total amount did not exceed 500 ml, were centrifuged to obtain a pellet. The cells were then resuspended in RPMI 1640 (5×10⁶ cells/ml) and layered on a two-step density gradient consisting of 15 ml of 100% (density: 1.077 g/ml) Lymphoprep (Axis-Shield, Oslo) overlaid with 15 ml of 75% Lymphoprep (diluted with RPMI 1640, at a density of 1.057 g/ml) and centrifuged (400 g, for 35 min at 24°C) to separate leukocyte mononuclear cells (MNC) from cancer and mesothelial cells [6].

Peripheral blood mononuclear cells (PBMC) were isolated by standard one-step density gradient centrifugation (1.077 g/ml). All cell fractions were washed, resuspended in RPMI and adjusted to the desired concentration.

Flow cytometry

Staining of 10⁶ cells/ml of PCF leukocyte MNC and PBMC was carried out with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated CD45 and CD14 (lymphocyte gating),

CD45RO, CD4, CD28 and HLA-DR monoclonal antibodies (Becton-Dickinson), read in the lymphocyte gate via FACStar⁺ (Becton-Dickinson) and analysed with the use of PC-lysis software.

mRNA study of CD45RO[±] cells

Total RNA was isolated with the use of a guanidine isothiocyanate-phenol-chloroform extraction technique, then electrophoretically and spectrophotometrically assessed and used in 10-ng quantity for reverse transcription (RT) reaction (RT-PCR kit; Stratagene).

The amount of cDNA used for polymerase chain reaction (PCR) was estimated and standardised against expression of the housekeeping GAPDH gene. In brief, serial dilutions of cDNA were amplified in parallel with GAPDH and tested with cytokine/protein primers. The intensity of the electrophoretic band reflecting the amount of given cytokine/protein transcripts was assessed in all samples at the cDNA dilution that gave a similarly strong intensity of the GAPDH transcript band. This meant that cytokine/protein transcript readings were made at a cDNA dilution ranging from 1:1 to 1:10,000. The final assessment of transcript electrophoretic band intensity was as follows: (0): no band; (+): visible; (++): strong; and (+++): abundant signal.

cDNA was amplified (Biometra UNO II thermocycler) with primer pairs specific for p53, Ki67 (Stratagene, USA), bcl-2 [19], GAPDH, IL-2, IL-4, IL-10, IL-8 [20] and IP-10 (5'-TGG CAT TCA AGG AGT ACC TC-3', 5'-TGT AGG GAA GTG ATG GGA GA-3'; annealing temperature: 55°C). Twenty-five microlitres of reaction mixture contained cDNA dilution, 0.125 pM dNTP (Gibco), 0.5 U of Taq-polymerase (Gibco), PCR Tris-HCl buffer (pH 8.3) and 25 pM of 5' and 3' primers. PCR products (10 µl) were electrophoresed in 1.5% agarose gel and stained with ethidium bromide.

mRNA study of IL-6 in OC tissue

RT-PCR was performed as described above. The amount of IL-6 transcripts (band density) was expressed against GAPDH

Table 1. Patient characteristics

Therapy		Total	Group 1	Group 2	Group 3
			PAC*	PC**	Other [****]
Age	N	44	22	9	13
	Mean ± SEM	56.1 ± 8.0	56.5 ± 7.2	58.5 ± 8.6	53.8 ± 6.9
	Min-max	36–71	38–69	45–71	36–68
Histiotype (n)	Serous	23	10	3	10
	Mucous	4	1	3	–
	Endometrioid	7	5	–	2
	Undifferentiated	8	3	3	1
	Nesonephroidal	3	3	–	–
FIGO (n)	IC	1	–	1	–
	IIC	1	–	–	1
	IIIC	32	20	5	7
	IV	10	2	3	5
RD (n)	< 5 cm		9	1	
	> 5 cm		13	8	
Surgery (n)	Radical		10	1	
	Partial resection		8	4	
	Explorative		4	4	
	Without surgery		–	–	–
Survival	N		22	9	
	Mean ± SEM (days)		839 ± 631	454 ± 234	
	Fatal cases		17	8	
	Cured cases		5	1	

*PAC cisplatin 50 mg/m², doxorubicin 50 mg/m², and cyclophosphamide 500 mg/m², i.v. bolus day 1 every 3 weeks for 6 cycles

**PC cisplatin 75 mg/m², cyclophosphamide 750 mg/m², i.v. bolus day 1 every 3 weeks for 7 cycles

***Other: patients received high-dose chemotherapy or paclitaxel or failed to complete PAC/PC treatment and were not considered in the survival analysis

transcripts band density. With this approach, OC tissue homogenates with a density ratio of IL-6 transcripts to GAPDH transcripts below 0.5 and above 0.5 constituted groups considered as composed of high and low IL-6 gene expressing tumours, respectively.

Isolation of CD45RO populations

PBMC and neoplastic effusion lymphocytes were washed and suspended in PBS-Ca²⁺Mg²⁺ MACS buffer containing 10% ACD-A and 5% human serum albumin (ZLB, Berno, Switzerland) at a concentration of 10⁸ cells/ml, then labelled with anti-CD11b and anti-CD45RO MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and finally separated on a SuperMACS equipped with VS⁺ columns according to the manufacturer's instructions.

Immunohistochemical staining

Formalin-fixed paraffin-embedded sections from ovarian tumours were evaluated for the presence of infiltrating lymphocytes expressing CD45RO antigen and activation marker HLA-DR. In all samples, microwave heating (3x5 min at 750 W in 0.01 M citric buffer) was performed for antigen retrieval. Anti-CD45RO and anti-HLA-DR monoclonal antibodies (Dako, Carpinteria, Calif.) were incubated on the slides for 30 min. Subsequently three-step immunoperoxidase staining was carried out according to the manufacturer's instructions (Dako). Anti SDF-1 monoclonal antibody (clone K15C, IgG2a) was kindly provided by F. Arenzana-Seisdedos (Unité d'Immunologie Virale, Institut Pasteur, Paris, France). The presence of SDF-1 was visualised with the EnVision AP kit (Dako) according to the manufacturer's instructions.

Statistical analysis

Differences between groups were analysed by the Chi² test with Yates correction, Fisher's exact test, the *t*-test for paired samples, or the Mann-Whitney *U*-test. A value of *P* < 0.05 was considered as significant.

Survival curves were generated using the Kaplan-Meier method [9] and compared using the Cox-Mantel test. Survival durations were measured from the period starting from the end of chemotherapy to the time of death. Several factors were evaluated as possible prognostic indicators for overall survival. These were age, histology, treatment (CAP or CP), and the presence of residual disease. Prognostic factors were subjected to univariate and multivariate survival analyses. All variables found to have *P* < 0.1 by univariate analysis were considered as candidates for stepwise Cox regression analysis.

Results

Excessively high levels of CD45RO[±] cells and survival

Our previous study reported higher proportions of monocytes, B cells, CD4⁺, HLA-DR⁺ and CD45RO⁺ lymphocytes in PCF as compared to levels in the blood of OC patients [11].

In this paper, we focussed on the activity of CD45RO⁺ lymphocytes. The percentage of CD45RO⁺ lymphocytes in PCF was significantly correlated with the duration of survival (*r* = 0.6, *P* = 0.0003, *n* = 31; Fig. 1).

The analysis of individual patients revealed that in 22 out of 31 cases the percentage of CD45RO⁺ lymphocytes was higher in the PCF than in the blood, and these excessively high levels ranged from 2.0% to 59%. Again, the proportion of CD45RO⁺ cells in PCF, expressed as

a higher level compared to that in the blood, was associated with the duration of survival (*P* = 0.02; Fig. 2).

Clinical and laboratory parameters which may influence the association between the percentage of PCF CD45RO lymphocytes and survival

To exclude confounding factors, i.e. clinical parameters known to affect the association between survival and the percentage of CD45RO lymphocytes in PCF, a Cox

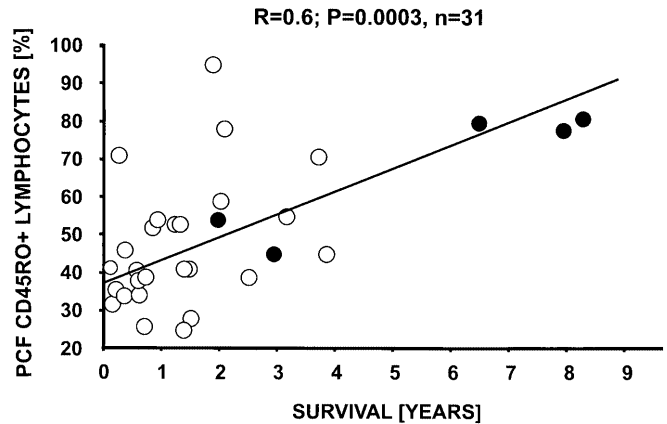


Fig. 1. Correlation between percentages of CD45RO⁺ lymphocytes in PCF and survival of OC patients (white and grey circles represent deceased subjects and patients who survived respectively)

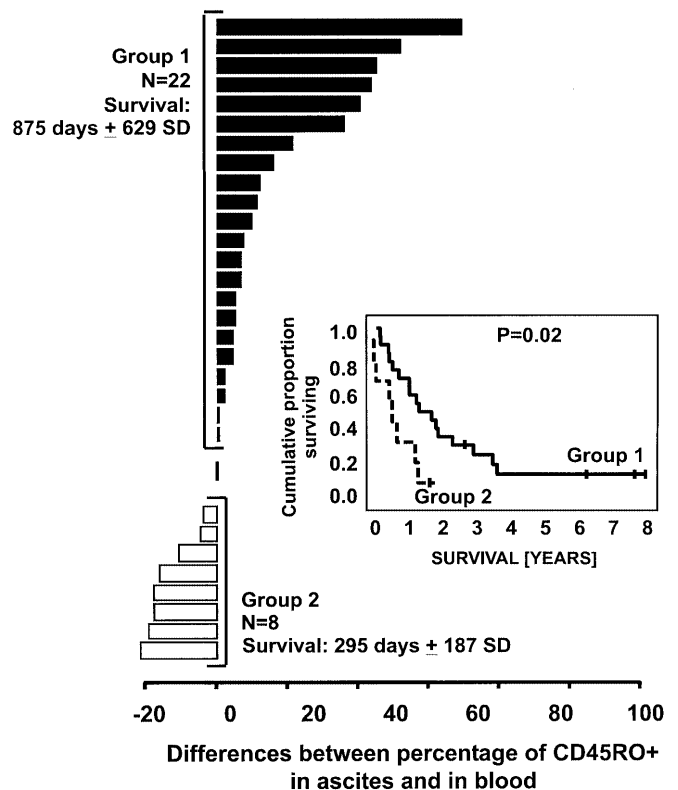


Fig. 2. Higher percentages of CD45RO⁺ lymphocytes in PCF compared to those in in blood with regard to survival. *Cox-Mantel test

analysis was performed. This included age, FIGO status, histology, treatment (CAP or CP), the presence of RD and PCF CD45RO⁺. In the univariate analysis, a fraction of PCF CD45RO⁺ lymphocytes, histopathology, RD and FIGO were found to be associated with survival ($P < 0.06$). All these parameters were further analyzed by multivariate analysis. The latter showed that only a fraction of the CD45RO⁺ cells ($RR: 0.97$, $P = 0.04$) and RD ($RR: 2.3$, $P = 0.01$) significantly affected the duration of survival.

Inoperable cases and patients with RD > 5 cm had a shorter survival duration than those with RD < 5 cm, but the CD45RO⁺ cell percentage in PCF was similar in patients with RD > 5 cm and in those with RD < 5 cm [41 ± 21 vs 38.5 ± 25 (median \pm SD); $P > 0.1$]. Patients with a high percentage of PCF CD45RO⁺ cells (above the median value of the whole group) had a longer duration of survival ($P = 0.0003$; Fig. 3); this was observed in subjects with RD below [71 ± 23 vs 33 ± 6 , n.s. (mean \pm SD)] and above 5 cm [62 ± 16 vs 25 ± 14 years (mean \pm SD), $P = 0.02$; Fig. 3].

IL-6 mRNA in tumour tissue

In the previous study, we showed that a positive correlation existed between PCF IL-6 activity and the percentage of CD45RO⁺ lymphocytes in neoplastic effusions [11]. This association was again tested in the present study at the IL-6 mRNA level. All patients were stratified according to the amount of IL-6 transcripts in tumour tissue assessed by semi-quantitation. Patients with a high IL-6 to GAPDH ratio had significantly higher serum CRP levels compared to the group of patients with a rather poor expression of the IL-6 gene (IL-6 to GAPDH ratio < 0.5; $P = 0.004$; Fig. 4). Similarly,

patients with a high IL-6 mRNA to GAPDH mRNA ratio had a higher proportion of CD45RO⁺ lymphocytes in their PCF compared to patients with a lower level of IL-6 gene expression in tumour tissue ($P = 0.006$; Fig. 4).

CD45RO-positive lymphocytes

Phenotype

PCF and blood CD45RO⁺ cells similarly co-expressed CD3 ($89\% \pm 14$ SD and $85\% \pm 11$ SD), but PCF cells were more frequently positive for CD4 (33.1 ± 2.5 vs 19.3 ± 1.8 SEM, $n = 9$, $P < 0.002$), CD28 (81 ± 25 vs 69 ± 19 SEM, $n = 8$, $P < 0.01$; Fig. 5) and HLA-DR (67 ± 18 vs 31 ± 13 SEM, $n = 8$, $P < 0.01$; Fig. 5).

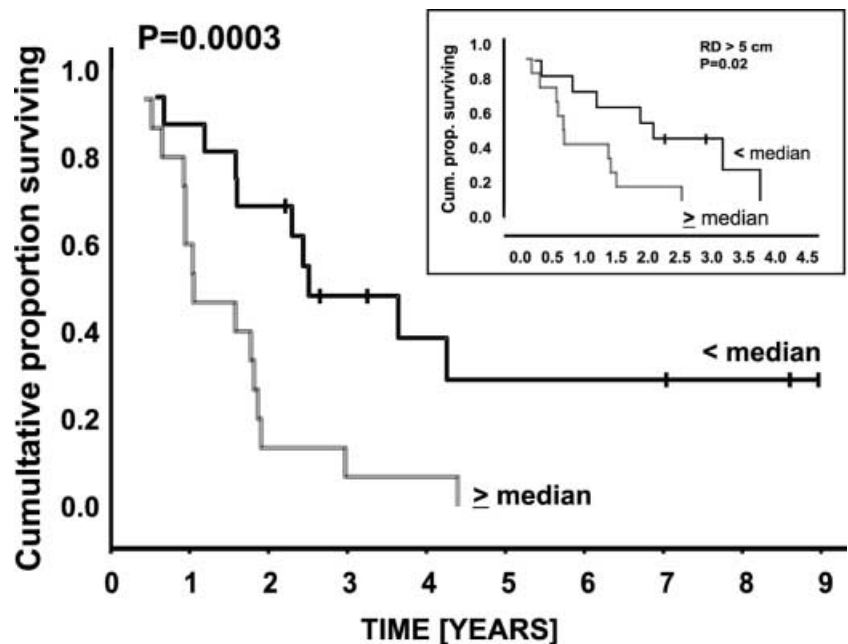
Gene expression

CD45RO⁺ lymphocytes purified from PCF possessed more p53 ($P = 0.01$, $n = 5$) and bcl-2 ($P = 0.03$, $n = 5$; Fig. 5) gene transcripts compared to those in the blood. The PCF CD45RO⁺ cells expressed higher levels of Ki67 than their blood counterparts ($P = 0.05$, $n = 5$; Fig. 5). In addition, memory lymphocytes from PCF expressed relatively high levels of IL-6 ($P = 0.01$), IL-10 ($P = 0.006$) and IP-10 ($P = 0.006$), but low levels of IL-2 ($P = 0.02$) compared to those in the peripheral blood (Fig. 6).

CD45RO, HLA-DR and SDF-1 antigen staining of tumour tissue

CD45RO⁺ cells were seen within the cancer tissue (10 out of 30 patients) and in the adjacent connective tissue

Fig. 3. Kaplan-Meier survival curve of OC patients with CD45RO⁺ lymphocyte percentages in PCF above (black line) and below (grey line) the median value of the whole group (45.5%); the upper corner shows survival in a subgroup of patients with RD > 5 cm. *Cox-Mantel test



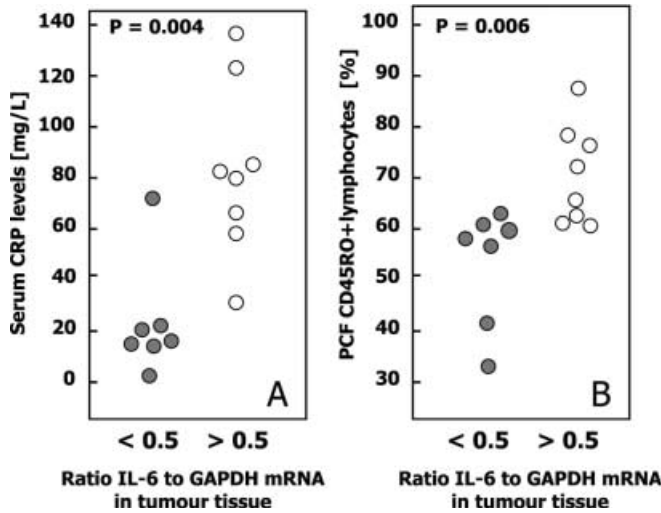


Fig. 4. Serum CRP levels (A) and percentage of CD45RO⁺ lymphocytes in PCF (B) in patients with higher and lower IL-6 gene expressing tumours. **t*-test for paired samples

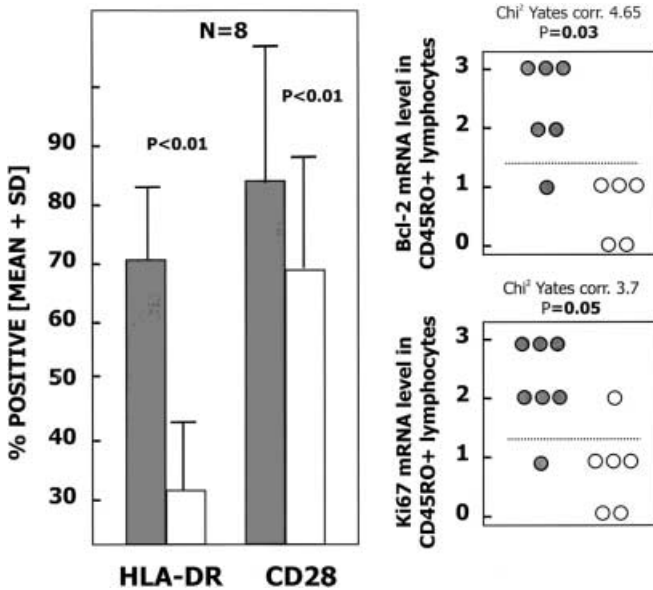


Fig. 5. HLA-DR⁺ and CD28⁺ cells and bcl-2 and Ki67 gene expression in CD45RO⁺ lymphocytes in PCF (grey shapes) and blood (white shapes); **t*-test for paired samples

(*n* = 21). In 3 cases this infiltration was more apparent, and the cells formed clusters; all these patients had a PCF CD45RO⁺ cell fraction above the mean + 1 SD of the whole group.

Aberrant HLA-DR antigen expression in cancer cells was seen in 11 out of 16 cancer tissue samples. This was not, however, associated with CD45RO⁺ cell infiltration in cancer tissue, or with the percentage of CD45RO⁺ cells in PCF.

Twenty cancer tissue samples were stained for the presence of SDF-1. Thirteen specimens showed positive staining, but in 9 cases only a proportion of the cells stained positive, and this staining was rather weak. Only in 4 tissue samples did all cells stain strongly positive. Of

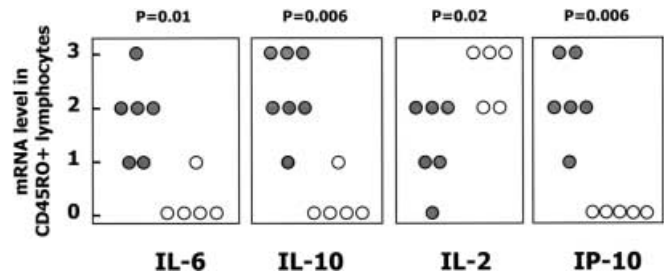


Fig. 6. IL-6, IL-2, IL-10 and IP-10 mRNA levels (accordingly to band intensity via electrophoresis of PCR products) in CD45RO⁺ lymphocytes obtained from PCF (grey circle) and blood (white circle); *Mann Whitney *U*-test

note is that in 3 out of these 4 cases and in only 2 out of 16 cases lacking or with only weak SDF-1 positivity the PCF CD45RO⁺ cell percentage was above the mean + 1 SD of the whole group (*P* = 0.03; Fisher's exact test).

Discussion

The survival of patients with OC is affected by the disease stage at presentation [15] and the effectiveness of surgery [16]. The latter aspect was also observed in the present study (Fig. 3). Chemotherapy is routinely used in more advanced cases. The response depends on the dose administered [14, 17], and the use of anthracyclines can also be beneficial [7]. Thus in our patient population, no statistical difference was found in survival between patients receiving PAC and those given PC. This might be due to the fact that PC patients received a higher cumulative dose of cyclophosphamide and cisplatin. Therefore, the lack of doxorubicin was counterbalanced by a higher cumulative dose of both cyclophosphamide and platinum. Independently of the clinical parameters, the proportion of CD45RO⁺ cells in PCF significantly influenced survival. This was seen in patients receiving PAC or PC (not shown) and with an RD > 5 cm (Fig. 3). To determine the significance of the disease stage at presentation, the following criteria were examined: RD post-surgery, type of chemotherapy, and percentage of CD45RO⁺ cells required for survival; univariate and multivariate analysis were then performed. Univariate analysis showed that the tumour load, RD and the percentage of CD45RO⁺ cells in PCF indicated a significant association with survival. The histopathology was only moderately associated (*P* = 0.06) with survival, and the criteria of age and chemotherapy regimens were not valid (*P* > 0.1), and could be excluded from any further analysis. The multivariate analysis demonstrated that only two independent factors, RD and the percentage of CD45RO⁺ cells in PCF, were significantly associated with survival.

Importantly, the presence of higher levels of CD45RO⁺ cells in PCF compared to those in the blood were of benefit to survival (Fig. 2). Therefore, in OC a mechanism appears to be involved that attracts

CD45RO⁺ cells to the vicinity of the tumour and it is this accumulation that favours survival (Fig. 3). PCF CD45RO⁺ lymphocytes, in contrast to those in the blood, were CD28⁺ and HLA-DR⁺, and expressed bcl-2 and Ki67 genes (Fig. 5). The analysis of cytokine gene expression suggested that they were Th2 in origin (positive for IL-10 but not IL-2) and met the criteria of lymphokine or antigen or activated T memory cells. Indeed, PCF CD45RO⁺ cells used only a restricted number of TCR V beta families (our unpublished findings). This suggests that CD45RO⁺ lymphocytes may accumulate locally, and proliferate in response to tumour-associated antigen. The HLA-DR positivity of epithelial cells enables them to present self-antigens [12].

Sixty-five percent of OC tumours analysed in the present study were HLA-DR-positive, but there was no association between this positivity and the accumulation of CD45RO⁺ cells in tumour tissue or in PCF. Therefore, an aberrant expression of HLA-DR antigen in the tumour tissue was unlikely to be responsible for the accumulation and triggering of memory T cells in PCF. Similarly, Allavena et al. failed to find an association between the HLA-DR positivity of OC cells and blastogenic response in a mixed lymphocyte tumour culture [3]. The other possibility was that chemokines could have played a role in PCF lymphocyte accumulation. This hypothesis was supported by the present study, which examined the association between the strong positivity of SDF-1 cancer tissue and the accumulation of CD45RO⁺ cells in PCF. Also a strong expression of the IP-10 gene was found in PCF lymphocytes. SDF-1 and IP-10 attract memory T cells [4]. Therefore, primarily (SDF-1 cancer cell expression) or secondarily (IP-10 production by activated lymphocytes) produced chemokines might play a role in CD45RO⁺ cell PCF accumulation.

The primary role might be played by IL-6 produced by OC which modulates the local immune response toward the Th2-type response. Keever-Taylor et al. [10] showed that rIL-6 administered to patients activated Th2-like T cells. This response includes cytokines with chemoattractant activity. Locally produced IP-10, the mRNA of which was found in PCF CD45RO⁺ cells (Fig. 6), may augment CD4⁺ cell influx [23, 24] and IL-6 together with TNF alpha and IL-2 induce the proliferation of CD45RO⁺ cells [25]. Local proliferation together with TNF alpha and IL-2 induce the proliferation of CD45RO⁺ cells [25].

The present study has examined the accumulation of CD45RO⁺ cells in PCF. The process of accumulation was associated with the cancer tissue production of SDF-1 and IL-6. PCF memory T cells with Th2 characteristics were activated, and the influx of CD45RO⁺ cells could be further augmented by the local production of IP-10. The direct interaction between activated memory T cells and cancer tissue has not been described previously, but it appears that the extent of CD45RO⁺ cell accumulation in PCF has a positive effect on survival.

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