Aim of the study: A number of observations have indicated that the immune system plays a significant role in patients with epithelial ovarian cancer (EOC). In cases of EOC, the prognostic significance of tumour infiltrating lymphocytes has not been clearly explained yet. The aim is to determine the phenotype and activation molecules of cytotoxic T cell and NK cell subpopulations and to compare their representation in malignant ascites and peripheral blood in patients with ovarian cancer.

Material and methods: Cytotoxic cells taken from blood samples of the cubital vein and malignant ascites were obtained from 53 patients with EOC. Their surface and activation characteristics were determined by means of a flow cytometer. Immunophenotype multiparametric analysis of peripheral blood lymphocytes (PBLs) and tumour infiltrating lymphocytes (TILs) was carried out.

Results: CD3+ T lymphocytes were the main population of TILs (75.9%) and PBLs (70.9%). The number of activating T cells was significantly higher in TILs: CD3+/69+ 6.7% vs. 0.8% (p < 0.001). The representation of (CD3+/16+56+) NK cells in TILs was significantly higher: 11.0% vs. 5.6% (p = 0.034), likewise CD56bright from CD56+ cells were higher in TILs and PBLs.

Conclusions: These results prove that the central effector role of T cytotoxic lymphocytes and natural killer (NK) cells in an anti-tumour response is well known [3–6]. However, objective findings indicate that tumour cells grow in the presence of mononuclear cell infiltration. These studies suggest that tumour-associated lymphocytes are not capable of evoking an effective immune response [7]. It is clear that the plasticity of tumour cells in changing the expression of cell-surface molecules, for example downregulation of human leukocyte antigen (HLA) molecules, or low density or even absence of tumour-specific antigens, is an important cause of the failure of the anti-tumour response. Cytotoxicity of T cells depends on the expression of HLA class I molecules. These properties mediate a negative effect on immune surveillance. Tumours can also fail to present their antigens owing to defects in the intracellular peptide transporter TAP or in the components of immunoproteasome genes [8–10]. Besides the insufficient tumour immune response due to alteration of the processing cascade of a specific antigen, a great number of studies document the direct suppressive effect of the tumour environment on cytotoxic lymphocyte effectiveness [11]. Although cancer stroma lymphocyte infiltrations are present in the majority of human neoplasms, these lymphocytes are mostly functionally defective, incompletely activated and anergic [3]. Through the downregulation of activation and proliferation of tumour-specific cytotoxic T lymphocytes and NK cells, cancers are able to overcome immune surveillance. Incompletely activated tumour infiltrating lymphocytes (TILs) and cancer cells produce chemokines that are chemoattractants for other leu-
kocytes and regulate their migration into tumour stroma. The prognostic significance of TILs has been recently recognized in various neoplasms. In epithelial ovarian cancer, most of the studies performed in paraffin-embedded tissue have shown that the presence of TILs is an independent favourable prognostic factor. Numerous investigators have reported that TILs obtained from patients with ovarian carcinoma contain activated cytotoxic T lymphocytes and NK cells, which were subsequently ex vivo cultivated with a low concentration of IL-2. In these instances, the CD8+ T cell, TcR positive and NK cell population expanded, and the cells exhibited primarily autologous tumour cell cytotoxicity [4, 12, 13].

The presence of ascites in advanced stages of ovarian cancer offers a unique opportunity to obtain tumour-associated lymphocytes (TALs) directly from the site of a malignant process. There is still limited data about the prognostic significance of cytotoxic T cells and NK cell subtypes freshly isolated from a tumour or ascites [17, 18]. But some studies show that TALs may play an important role in the immune response against tumours [17].

For the above reasons, we began to study prospectively the population of cytotoxic cells in ascites associated with epithelial ovarian cancer concomitantly with peripheral blood lymphocytes. Here, we present the results of a flow cytometric phenotype analysis of both cytotoxic T cells and NK cell subtypes and their activation molecules in ascitic fluid and peripheral blood. Due to our better knowledge we have used a brighter spectrum of activation markers than before.

Material and methods

Patients

Ascites and peripheral blood were obtained from 53 patients with diagnosed ovarian cancer or patients undergoing surgery for suspected ovarian cancer prior to neo-adjuvant, adjuvant or palliative chemotherapy. The study was carried out between January 2006 and 2013 at the University Hospital Hradec Králové, Czech Republic. Inclusion in the study was confirmed after a histopathologic diagnosis of invasive epithelial ovarian carcinoma (Table 1). All patients gave their written informed consent prior to their participation in the study. The study was approved by the Institutional Review Committee. The median age of the patients was 64 (range 40–83). In 38 patients (71%), a radical operation was performed, although only 11 of the patients was 64 (range 40–83). In 38 patients (71%), the study was carried out between January 2006 and 2013 at the University Hospital Hradec Králové, Czech Republic. The median age of the patients was confirmed after a histopathologic diagnosis of invasive epithelial ovarian carcinoma (Table 1). All patients gave their written informed consent prior to their participation in the study. The study was approved by the Institutional Review Committee. The median age of the patients was 64 (range 40–83). In 38 patients (71%), a radical operation was performed, although only 11 of them (29%) received neo-adjuvant chemotherapy. The most frequent tumour stage was FIGO IIIC (60%); in our most frequent tumour stage was FIGO IIIc (60%); in our most frequent tumour stage was FIGO IIIc (60%); in our

was 1996 days (25–2563) and at the end of follow-up on 1st January 2013, 22 (41%) were still alive.

Methods

Heparinized test tubes were used to collect peripheral blood from the cubital vein. The malignant ascites were obtained via open surgery, laparoscopy or paracentesis before chemotherapy in tubes with a heparin solution in a final concentration of 25 U/ml. The ascitic fluids were centrifuged at 1000 rpm for 10 minutes at 4°C. Isolated cells were twice washed and diluted to an approximate concentration of 5 × 10⁴ cells. The samples were processed up to 2 hours after their sampling. Each time, 25 µl of heparinized blood and cellular suspension were incubated for 20 minutes in the dark at room temperature with 10 µl of diluted conjugated monoclonal antibodies (Immunotech and Beckman Coulter). The combinations were as follows: CD45-FITC/CD14-PE (for gating), CD3-FITC/CD16/56-PE/CD19-PC5, CD45RO-FITC/CD45RA-PE/CD19-PC5, CD56-PE/NKG2D-FITC and CD3-FITC/CD8-PE/TcRαβ-PE. After lysing erythrocytes with a lysing solution (0.5 ml of OptiLyse C, Beckman Coulter), a buffer was added with 0.5% foetal calf and 0.01% sodium azide. The measurement was carried out by means of a three-colour fluorescence Coulter Epics XL flow cytometer (the Coulter Company, Fullerton, USA). At least 5000 gated events were analysed. Subpopulations were measured as a percentage of the total number of CD45-positive cells (and the total number of CD3-positive cells). The expression of activation markers CD69, HLA-DR and CD25 molecules was assessed on gated CD3 positive cells by plotting CD4 or CD8 positive cells vs. the given activation markers. The evaluation of measured samples was carried out by means of CPX analysing software (Fig. 1). The results were calculated from the data of 53 patients suffering from EOC.

Statistical evaluation of the measured values was carried out by means of NCSS 2007 Statistica. The statistics were processed using standard methods for a statistical comparison of two groups. Normality value tests were unsuccessful in the evaluated cases; for this reason, the one-way ANOVA on ranks test was carried out to determine the level of statistical significance. The median was an indicator of the value position. Using Cox proportional-hazards regression, we observed the potential effect of acquired immunological parameters from ascitic fluid, both on the overall survival, and also on the length of time to first progression. Differences were considered significant at p < 0.05.

Results

The phenotype of cytotoxic cells was determined by means of the three-colour immunofluorescence method, and their representation in peripheral blood (PB) and ascites was compared. The both compartments exhibited a dominant CD3+ T lymphocyte population. The number of CD3+/CD69+ (activated) cells was significantly higher in ascites (p < 0.001). The CD8+ T lymphocyte count was...
Table 1. Clinical and histopathological characteristics of patients with ovarian carcinoma

| Patient number | Age (years) | FIGO classification | Histology | Grade | Radical surgery | Neoadjuvant treatment | Number of treatment lines | Time to first progression (days) | Overall survival (days) |
|----------------|-------------|---------------------|-----------|-------|-----------------|-----------------------|---------------------------|----------------------------------|------------------------|
| 1              | 60          | IIIC                | serous    | 1     | no              | yes                   | 3                         | 343                              | 986                    |
| 2              | 56          | IIIC                | serous    | 2     | yes             | yes                   | 2                         | 0                                | 2315                   |
| 3              | 53          | IIIC                | serous    | 3     | yes             | no                    | 5                         | 719                              | 2267                   |
| 4              | 62          | IIIC                | serous    | 3     | yes             | yes                   | 3                         | 993                              | 1367                   |
| 5              | 51          | IV                  | serous    | 3     | no              | no                    | 1                         | no                               | 134                    |
| 6              | 55          | IIIC                | serous    | 3     | yes             | yes                   | 5                         | 640                              | 2374                   |
| 7              | 47          | IIIC                | serous    | 3     | yes             | no                    | 5                         | 1211                             | 2395                   |
| 8              | 69          | IIIC                | serous    | 3     | no              | no                    | 2                         | 217                              | 754                    |
| 9              | 62          | IIIC                | serous    | 2     | yes             | no                    | 0                         | 0                                | 2228                   |
| 10             | 65          | IIIC                | serous    | 3     | yes             | no                    | 4                         | 489                              | 2093                   |
| 11             | 61          | IIIC                | serous    | 3     | yes             | yes                   | 1                         | 0                                | 2262                   |
| 12             | 70          | IIIC                | serous    | 3     | yes             | yes                   | 5                         | 589                              | 1321                   |
| 13             | 74          | IA                  | undifferentiated | 2     | yes             | no                    | 0                         | 0                                | 2492                   |
| 14             | 69          | IIIC                | endometrioid | 3   | yes             | no                    | 1                         | 0                                | 2190                   |
| 15             | 69          | IC                  | endometrioid | 3   | yes             | yes                   | 2                         | 452                              | 615                    |
| 16             | 71          | IV                  | serous    | 3     | no              | no                    | 1                         | no                               | 89                     |
| 17             | 64          | IIIC                | serous    | 3     | yes             | no                    | 2                         | 580                              | 963                    |
| 18             | 82          | IIIC                | endometrioid | 3   | yes             | no                    | 1                         | 0                                | 338                    |
| 19             | 64          | IIIC                | serous    | 3     | yes             | yes                   | 2                         | 450                              | 529                    |
| 20             | 55          | IIIA                | endometrioid | 2   | yes             | no                    | 5                         | 938                              | 1222                   |
| 21             | 51          | IIIC                | undifferentiated | 3   | yes             | no                    | 2                         | 0                                | 2169                   |
| 22             | 78          | IIIB                | serous    | 3     | no              | no                    | 1                         | NO                               | 267                    |
| 23             | 65          | IIIC                | serous    | 3     | yes             | no                    | 1                         | 0                                | 2116                   |
| 24             | 83          | IIIIC               | endometrioid | 2   | no              | no                    | 2                         | 377                              | 583                    |
| 25             | 40          | IIIA                | serous    | 1     | yes             | no                    | 1                         | 0                                | 2499                   |
| 26             | 64          | IIIC                | serous    | 3     | yes             | no                    | 1                         | 0                                | 2190                   |
| 27             | 51          | IV                  | serous    | 3     | yes             | no                    | 1                         | 0                                | 2563                   |
| 28             | 71          | IIIC                | serous    | 3     | yes             | yes                   | 4                         | 854                              | 2041                   |
| 29             | 57          | IIIB                | endometrioid | 2   | yes             | no                    | 1                         | 0                                | 2116                   |
| 30             | 74          | IIIA                | serous    | 3     | no              | no                    | 4                         | 102                              | 2081                   |
| 31             | 67          | IIIC                | serous    | 3     | no              | no                    | 0                         | no                               | 28                     |
| 32             | 79          | IIIC                | serous    | 3     | no              | no                    | 0                         | no                               | 63                     |
| 33             | 77          | IIIC                | endometrioid | 2   | no              | no                    | 0                         | no                               | 54                     |
| 34             | 56          | IIIA                | serous    | 3     | yes             | no                    | 5                         | 703                              | 1996                   |
| 35             | 72          | IIIC                | undifferentiated | 3   | no              | no                    | 0                         | 114                              | 707                    |
| 36             | 51          | IIIC                | serous    | 3     | yes             | no                    | 0                         | 0                                | 2224                   |
| 37             | 71          | IIIC                | mucinous  | 3     | yes             | no                    | 3                         | 185                              | 593                    |
| 38             | 59          | IIIA                | serous    | 2     | yes             | no                    | 3                         | 279                              | 2268                   |
| 39             | 56          | IIIB                | serous    | 3     | yes             | no                    | 0                         | 0                                | 2002                   |
| 40             | 54          | IIIA                | endometrioid | 3   | yes             | no                    | 1                         | 428                              | 1761                   |
| 41             | 72          | IIIA                | undifferentiated | 3   | yes             | no                    | 1                         | 0                                | 2122                   |
| 42             | 61          | IIIB                | undifferentiated | 3   | yes             | no                    | 0                         | 131                              | 907                    |
| 43             | 74          | IIIC                | serous    | 3     | yes             | no                    | 1                         | 0                                | 2190                   |
| 44             | 66          | IIIB                | endometrioid | 2   | yes             | yes                   | 5                         | 440                              | 1596                   |
| 45             | 72          | IIIb                | mucinous  | 3     | no              | no                    | 0                         | 0                                | 25                     |
| 46             | 68          | IIIC                | undifferentiated | 3   | no              | no                    | 0                         | no                               | 53                     |
| 47             | 48          | IIIA                | serous    | 2     | yes             | no                    | 1                         | 0                                | 2105                   |
| 48             | 66          | IIIC                | serous    | 2     | yes             | no                    | 1                         | 0                                | 2122                   |
| 49             | 72          | IIIC                | endometrioid | 3   | yes             | yes                   | 2                         | 259                              | 589                    |
| 50             | 61          | IV                  | serous    | 3     | no              | no                    | 0                         | no                               | 130                    |
| 51             | 66          | IIIC                | serous    | 2     | no              | no                    | 0                         | no                               | 53                     |
| 52             | 62          | IIIC                | serous    | 3     | yes             | no                    | 1                         | 1711                             | 2240                   |
| 53             | 59          | IIIC                | serous    | 3     | yes             | yes                   | 3                         | 154                              | 2292                   |
Table 2. Phenotyping analysis of CD45 positive cells from peripheral blood and malignant ascites from 53 patients with ovarian cancer

| Cell population     | Peripheral blood (%) | Ascites (%) | \( p \) |
|---------------------|----------------------|-------------|--------|
| CD3+                | 70.9 (61.8–75.1)     | 75.0 (71.1–82.4) | 0.097 |
| CD3+/CD69+          | 0.8 (0.2–2.1)        | 6.7 (4.5–15.2) | < 0.001 |
| CD3+/CD25+          | 4.9 (3.6–5.8)        | 3.7 (2.4–5.3) | 0.381 |
| CD3+/DR+            | 1.4 (0.7–2.8)        | 4.0 (1.1–7.0) | 0.068 |
| CD3+/CD4+           | 48.7 (39.1–57.1)     | 49.1 (34.1–52.0) | 0.446 |
| CD4+/CD38+          | 8.5 (2.7–11.0)       | 2.6 (1.3–5.7) | 0.007 |
| CD4+/CD57+          | 30.6 (19.2–34.2)     | 37.7 (25.3–45.1) | 0.013 |
| CD8+                | 21.1 (15.7–24.8)     | 30.0 (20.3–36.6) | 0.033 |
| CD3+/CD8+           | 19.1 (17.1–20.8)     | 28.7 (16.8–33.2) | 0.012 |
| CD8+/CD38+          | 2.9 (1.2–6.5)        | 3.9 (3.0–4.9) | 0.205 |
| CD8+/DR+            | 0.6 (0.3–1.4)        | 2.3 (0.7–5.3) | < 0.001 |
| CD3–/CD8+           | 25.6 (8.8)           | 37.4 (19.9–52.6) | 0.065 |
| CD56+               | 2.1 (0.6–4.8)        | 1.5 (0.2–3.9) | n.s.  |
| CD3–/CD16+          | 2.5 (1.9–7.4)        | 10.3 (7.0–25.6) | < 0.001 |
| CD56+ from CD38+    | 45.9 (31.5–73.5)     | 83.2 (53.7–92.4) | < 0.001 |
| CD56dim 16+         | 1.9 (0.9–5.8)        | 8.25 (3.8–16.3) | < 0.001 |
| NKG2D+              | 4.4 (1.4–7.8)        | 1.7 (0.6–6.9) | 0.021 |
| NKG2D+ from CD56+   | 32.3 (25.2–37.6)     | 45.05 (30.9–58.1) | 0.034 |
| CD8+/CD56+          | 5.8 (3.2–9.5)        | 7.7 (3.75–10.55) | n.s. |

Table 3. Expression of membrane molecules on the NK cell subsets

| Cell population                   | Peripheral blood (%) | Ascites (%) | Significance |
|-----------------------------------|----------------------|-------------|--------------|
| CD3–/CD16+/CD56+                 | 5.6 (2.7–10.7)       | 11.0 (7.1–12.5) | 0.041        |
| CD56+ from CD38+                 | 5.5 (1.2–7.2)        | 3.1 (0.6–4.8) | 0.080        |
| CD56+ from CD38+                 | 11.7 (7.7–13.8)      | 12.9 (5.1–15.5) | n.s.         |
| CD53+ from CD38+                 | 8.2 (3.1–12.5)       | 12.1 (7.8–15.1) | 0.036        |
| CD56+ from CD16+                 | 2.1 (0.6–4.8)        | 1.5 (0.2–3.9) | n.s.         |
| CD56bright                       | 2.5 (1.9–7.4)        | 10.3 (7.0–25.6) | < 0.001      |
| CD56bright from CD56+            | 45.9 (31.5–73.5)     | 83.2 (53.7–92.4) | < 0.001      |
| CD56bright 16+                   | 1.9 (0.9–5.8)        | 8.25 (3.8–16.3) | < 0.001      |
| CD56dim 16+                      | 4.4 (1.4–7.8)        | 1.7 (0.6–6.9) | 0.021        |
| NKG2D+                           | 32.3 (25.2–37.6)     | 45.05 (30.9–58.1) | 0.034        |
| NKG2D+ from CD56+                | 89.9 (85.0–93.1)     | 91.0 (86.5–95.0) | n.s.         |
| CD8+/CD56+                       | 5.8 (3.2–9.5)        | 7.7 (3.75–10.55) | n.s.        |

n.s. – not significant

The results of NK cell analysis are presented in Table 3. Under physiological conditions, NK lymphocytes represent 10–15% of the lymphocytic population [14]. In patients with advanced EOC, counts of NK cells in peripheral blood (5.6%) were significantly lower (\( p = 0.041 \)) than those in ascites (11.0%). In both samples, NK cells were gated from the mononuclear CD45+/CD3+ population. The CD56bright subpopulation was determined in the lymphocyte and NK cell population. In both cases, significantly high-
representation of CD56<sup>bright</sup> NK cells was ascertained in the ascitic fluid. In contrast, the representation of CD56<sup>dimm</sup> CD16<sup>+</sup> NK cells was significantly higher in peripheral blood. No differences were found in the expression of the natural-killer group 2, member D (NKG2D) activation receptor in the population of NK cells; a significantly higher count of lymphocytes isolated from ascites was expressed by the NKG2D receptor ($p = 0.034$).
Comparative study of various subpopulations of cytotoxic cells in blood and ascites from patients with ovarian carcinoma

The influence of various immunological cells in ascitic fluid on overall survival was calculated with a multivariate model using a coefficient of determination of 0.65 with a significance level of \( p < 0.001 \) (Tables 4, 5). The presence of lymphocytes with the surface marker CD4 in ascitic fluid led to an increased risk of death with a hazard ratio (HR) of 1.2127 (95% CI: 1.0384–1.4162). When all the lymphocyte subsets were examined, we found that the presence of CD3–/CD16+/CD56+ and CD43+/CD8+ lymphocytes in ascitic fluid indicated a reduced risk of death with an HR of 0.6917 (95% CI: 0.5434–0.8804) and 0.8449 (95% CI: 0.603–1.192), respectively. But in the case of CD3–/CD8+ and CD3+/CD16+/CD56+ lymphocytes, the trend was reversed, with an HR of 1.3139 (95% CI: 1.010–1.6909) and 1.3350 (95% CI: 1.0788–1.6520), respectively. For other cell subsets no impact on survival was found.

In the case of the impact of the observed immunological cells in ascitic fluid on time to the first progression, we did not observe any correlation or impact.

**Discussion**

Ascites in cancer patients can reflect different pathogenic mechanisms of development. Peritoneal carcinomatosis is the most common cause of malignancy-related ascites, followed by massive liver metastases and the obstruction of lymph nodes and vessels. Lymphocytes present in the ascites were consistently referred to as TILs. In the case of malignancy-related ascites not associated with peritoneal carcinomatosis, the term “tumour-associated lymphocytes” (TALs) is much more suitable. Unfortunately, in individual cases it is difficult to identify prevailing mechanisms of ascites formation. The progression of cancer in the peritoneal cavity and the frequent formation of ascites, which characterize the advanced stage of ovarian cancer, make this tumour a model for the study of different lymphocytic populations [15].

Studies comparing the representation of cellular populations in tumour-induced ascites fluid with findings of ascites for other reasons can be reproduced and interpreted only with difficulty because no “normal” ascites exist. It is always a pathologic finding induced by some of the above-mentioned mechanisms that possesses an inflammatory component of various intensity, e.g. cirrhosis [16, 17].

The presence of tumour-infiltrating lymphocytes in the EOC stroma is important in terms of prognosis, which is confirmed by the outcomes of numerous studies [19, 20]. In our study, obtained data from Cox proportional-hazards regression describing the relationship between each immunological cellular subset and overall survival or time to progression needs to be interpreted with caution due to the heterogeneity of the studied population (Figs. 2, 3). But it shows that the presence of NK cells and cytotoxic T cells in ascitic fluid may play a positive role.

CD3+ T lymphocytes are the main cellular population situated at the site of the tumour as well as peripheral blood. The number of CD3+CD4+ lymphocytes dominated the CD3+/CD8+ lymphocytes and did not differ between the two compartments studied. This result is in compliance with a study by Bamias et al.; nevertheless, in the case of

| Table 4. Influence of various immunological cells in ascitic fluids on time to first progression using multivariate model with coefficient of determination of 0.49 with a significance level of \( p < 0.007 \) |
|------------------|--------|---------|------|
| CD4+RO          | 0.996  | 0.911–1.089 | 0.933 |
| CD4+RA          | 1.098  | 0.912–1.323 | 0.314 |
| CD3+CD69+       | 0.986  | 0.896–1.085 | 0.771 |
| CD8+CD57+       | 1.157  | 0.796–1.682 | 0.448 |
| CD3+CD25+       | 1.288  | 0.903–1.837 | 0.156 |
| CD8+DR+         | 0.869  | 0.371–2.037 | 0.748 |
| CD3+DR+         | 1.057  | 0.510–2.192 | 0.882 |
| CD3+CD16+CD56+  | 0.945  | 0.668–1.337 | 0.743 |
| CD3+CD16+CD56+  | 0.907  | 0.717–1.147 | 0.406 |
| CD3+CD8+        | 1.160  | 0.904–1.489 | 0.256 |
| CD3–CD8+        | 1.011  | 0.900–1.135 | 0.857 |
| CD19+           | 1.085  | 0.943–1.248 | 0.239 |
| CD8+            | 0.982  | 0.892–1.081 | 0.709 |
| CD4+            | 1.016  | 0.930–1.109 | 0.729 |
| CD3+            | 1.060  | 0.957–1.174 | 0.264 |

| Table 5. Influence of various immunological cells in ascitic fluids on overall survival using multivariate model with coefficient of determination of 0.65 with a significance level of \( p < 0.001 \) |
|------------------|--------|---------|------|
| CD4+RO          | 0.954  | 0.877–1.037 | 0.272 |
| CD4+RA          | 1.037  | 0.924–1.164 | 0.539 |
| CD3+CD69+       | 1.074  | 0.967–1.193 | 0.173 |
| CD8+CD57+       | 1.092  | 0.789–1.153 | 0.596 |
| CD3+CD25+       | 0.848  | 0.603–1.192 | 0.330 |
| CD8+DR+         | 0.897  | 0.332–2.424 | 0.830 |
| CD3+DR+         | 1.066  | 0.472–2.411 | 0.878 |
| CD3+CD16+CD56+  | 1.335  | 1.079–1.652 | 0.014 |
| CD3+CD16+CD56+  | 0.692  | 0.543–0.880 | 0.003 |
| CD3+CD8+        | 1.314  | 1.021–1.691 | 0.044 |
| CD3–CD8+        | 0.850  | 0.753–0.959 | 0.002 |
| CD19+           | 1.085  | 0.947–1.243 | 0.225 |
| CD8+            | 1.021  | 0.927–1.123 | 0.674 |
| CD4+            | 1.213  | 1.038–1.416 | 0.010 |
| CD3+            | 1.000  | 0.895–1.119 | 0.995 |
The cytotoxic reaction as ensured by cytotoxic CD8\(^+\) T lymphocytes and NK cells is crucial for the achievement of an effective anti-tumour response. In the majority of CD3\(^+\) and CD8\(^+\) T lymphocytes, the receptor for antigen (TcR) is created by the \(\alpha\beta\) heterodimer, and the relationship between TcR \(\alpha\beta\) and TcR\(\gamma\delta\) lymphocytes is the same both in the peripheral blood and in ascites [25, 26]. Our findings, along with those from experimental studies, have indicated that the specific anti-tumour response is not restricted by the TcR type [27, 28]. The relative count of CD3\(^+\)/CD8\(^+\) lymphocytes was significantly higher in ascites. Under physiological conditions, the CD8\(^+\) population expressing the CD56 and CD57 molecule (NK type of T lymphocytes) is a minority population in peripheral blood; however, its presence is higher in lymphatic nodes, the spleen and bone marrow. The NK type CD8\(^+\) lymphocytes exhibits an oligoclonal expansion \(\alpha\beta\) of the TcR chain different from other CD8\(^+\) T lymphocytes after antigenic stimulation. Based on this fact, CD56\(^+\) and CD57\(^+\) T lymphocytes are considered an independent population of cytotoxic lymphocytes. Their role is not known precisely, however [29, 30]. These lymphocytes of CD8\(^+\) NK type produce IFN-\(\gamma\) more intensively than CD8\(^+\) T lymphocytes [31]. The count of CD8\(^+\)/57\(^+\) cytotoxic lymphocytes, although not achieving significance, was lower in the ascitic fluid of our patients than in their peripheral blood. Such a reduction can be caused both by insufficient proactivation and strengthened inhibition signals and, subsequently, by an enhanced switch of activated cytotoxic cells into apoptosis [29]. The switch of stimulated cells into apoptosis takes place in the absence of accessory intercellular bindings, but apoptosis can also be induced by soluble factors from the family of TNF proteins produced by cells of the tumour stroma, mainly macrophages associated with the tumour [32–34]. Activated T lymphocytes express CD69 and HLA-DR membrane molecules. The early activation antigen CD69 belongs to the superfamily of C-type lectin. Following activation of T lymphocytes, it is one of the first proteins expressed within the membrane. It is involved in strengthening intercellular interactions as a co-stimulation molecule. The CD69 molecule is not line-specific and is also situated on other blood cells [35]. The finding of a significantly higher representation of CD3\(^+\)/CD69\(^+\) cells in ascites than in peripheral blood indicates a stimulation of T lymphocytes in which the cytokine/chemokine microenvironment is also involved together with tumour antigens. The enhanced representation of CD3\(^+\)/DR\(^+\) lymphocytes in ascites did not reach statistical significance in our patients compared to peripheral blood, but it exhibited a similar trend as mentioned in the paper by Bamias et al., who observed a significantly higher representation of CD4\(^+\)/HLADR and CD8\(^+\)/HLADR of T lymphocytes in ascites [17]. The CD3\(^+\)/CD25\(^+\) population of lymphocytes was considered proof of the alpha-chain expression for IL-2 on T lymphocytes of another origin, for instance a non-small-cell lung carcinoma or renal carcinoma, the representation of CD4\(^+\) and CD8\(^+\) lymphocytic subpopulations was different [17, 19, 21]. The number of CD4\(^+\)/CD45RO\(^+\) lymphocytes in the ascites of our patients was significantly higher than in peripheral blood. Also, the ratio of CD4\(^+\) T lymphocytes which had undergone rearrangement of gene segments for TcR (CD45RO\(^+\)) to naïve (CD45RA\(^+\)) was higher in ascites. This finding can be considered proof of the activation for TcR (CD45RO\(^+\)) to naïve (CD45RA\(^+\)) was higher in ascites which had undergone rearrangement of gene segments for TcR (CD45RO\(^+\)) to naïve (CD45RA\(^+\)) was higher in ascites. This finding can be considered proof of the activation for TcR (CD45RO\(^+\)) to naïve (CD45RA\(^+\)) was higher in ascites. This finding can be considered proof of the activation for TcR (CD45RO\(^+\)) to naïve (CD45RA\(^+\)) was higher in ascites.
Selective uptake of CD56 bright NK cells by the CD62L-phatic nodes can contain up to 95% of CD56 bright NK cells. Even under physiological conditions, lymphatic drainage on suppression induced by the existence of EOC, the expression of mucin molecules (MUC) on tumour cells is important [45]. In particular, the presence of MUC16, the carrier for the CA125 tumour marker, is able to inhibit an efficient cytotoxic reaction as mediated by NK cells [6]. In compliance with data from literature resources, significantly higher counts of CD56 bright NK cells in ascites were also found in EOC patients' ascites in our female patients. The finding of a higher number of CD3+/CD25+ lymphocytes in peripheral blood of our female EOC patients was surprising to us, and we are going to subject it to further analysis.

The precise role of B lymphocytes in the anti-tumour response is not known. Their presence in the tumour stroma confirms their involvement in the anti-tumour response [38]. In the case of tumours, the antibody response to tumour antigens after Ig binding can lead to blockage of epitopes distinguished by specific cytotoxic T lymphocytes, and the tumour growth can be potentiated. Higher occurrence of B lymphocytes among TILs was observed in patients with prevailing activity of CD4+ infiltrating lymphocytes, respectively the Th2 population. The significance of the role of the antigen-presenting cell has not been explicitly demonstrated in the case of B lymphocytes in tumour diseases [39–43]. In our patients, the incidence of B lymphocytes in ascites was significantly lower than that in peripheral blood.

NK cells constituting an important component of the natural immunity play an essential role in the anti-tumour response. They are a significant producer of IFN-γ and TNF-α. Activation of NK cells and the cytotoxic response are not dependent on the preceding sensitization [44]. In experimental studies, NK cells exhibited cytotoxicity targeted against EOC cells after IL-2 activation. In the case of EOC, the expression of mucin molecules (MUC) on tumour cells is important [45]. In particular, the presence of MUC16, the carrier for the CA125 tumour marker, is able to inhibit an efficient cytotoxic reaction as mediated by NK cells [6]. In compliance with data from literature resources, significantly higher counts of CD56bright NK cells in ascites were also found in EOC patients' ascites in our case [46]. Such a finding can be caused by blockage of the lymphatic drainage on suppression induced by the expanding tumour. Even under physiological conditions, lymphatic nodes can contain up to 95% of CD56bright NK cells [47]. Selective uptake of CD56bright NK cells by the CD62L molecule expressed on NK cells in lymphatic nodes can be another mode of action. Based on the latest studies, it is understood that CD56dim and CD56bright cells represent various differentiation stages of NK lymphocytes, with the CD56dim cells representing a more mature type [48]. It is impossible to exclude the possibility that maturation of NK cells is blocked after encountering tumour antigens. This possibility follows from experimental observations where MUC16 positive tumour cells inhibited expression of the CD16 molecule on NK cells from healthy donors [49]. The cytotoxic activity of CD56bright CD16− NK cells is lower, although granules containing perforins and granzymes are abundantly present in cytoplasm [50]. The situation when the CD16− cells accounted for more than 75% of CD56bright ascitic NK cells in our female patients can be considered a manifestation of the inhibited NK-mediated cytotoxicity.

NKG2D is a type II trans-membrane-anchored C-type lectin-like receptor. In the homodimer form it can be found not only on NK cells but also on NKT cells, CD8+ cytotoxic T cells and γδT cells, and it has also been described on CD4+ T lymphocytes. It is a highly evolutionary conserved receptor that distinguishes ligands exhibiting homology with class I MHC molecules [51]. A short intracellular section of the NKG2D receptor has no signal motif; it is associated with other signal-transducing proteins in the trans-membrane section [52]. In addition, two isoforms of NKG2D have been described, which differ by the presence or absence of 13 amino acids in the N end of the cytoplasmic part of the molecule. The long NKG2D isoform binds exclusively with DAP10 adapter protein and recruits phosphoinositide kinase-3 (PI3K) and growth factor receptor-bound proteins 2 (Grb2) by means of YINM. The short NKG2D isoform is associated both with DAP10 and the DAP12 signal-transducing protein [53]. The cytoplasmic part of the DAP12 protein carries the immunoreceptor tyrosine-based activation motif (ITAM) phosphorylation, which leads to involvement of the zeta-chain-associated protein kinase 70 (Zap70) and spleen tyrosine kinase (Syk) signal cascade. Each NK-G2D homodimer is associated with two DAP10 and DAP12 homodimers. It is not known whether DAP10 and DAP12 can be simultaneously contained in the hexameric receptor complex; this would, however, lead to a marked amplification of signal paths [54, 55].

A higher NKG2D positive lymphocyte count was found in the ascites of EOC patients than in peripheral blood, with this count being at the level of significance. Nevertheless, the lymphocytic population studied also included B lymphocytes, which do not express the CD94 and CD4+ receptors (NKp30, NKp44 and NKp46) are responsible for Signals coming by means of NKG2C, NKG2D, DNAX accessory molecules of 1 (DNAM-1) receptors and natural cytotoxic receptors (NKp30, NKp44 and NKp46) are responsible for the activation. The CD16 molecule mediates Ab-dependent cellular cytotoxicity. The function of the co-stimulation of LFA-1 and 284 molecules is also important. The combination of activation and inhibition signals is decisive for the function of NK cells [58].

Although our quantitative analysis showed similarities with previous studies [24, 43], the aforesaid data, as well as our findings, have confirmed the present idea of EOC...
cells and cells of other tumours affecting both the expression of membrane receptors of cytotoxic cells and the expression of their ligands, whether directly or by means of soluble mediators. The combination of chemotherapy and immunotherapy is still under consideration, and therefore molecules such as NKG2D still remain at the centre of interest. Although treatment using recombinant IFN-α and IFN-γ in clinical practice leads to strengthening the NK-G2D expression on cytotoxic cells, at the same time down-regulation of its HP60 ligand takes place on sarcoma cells, or of the MICA ligand on melanoma cells, thereby facilitating their evasion [56, 59]. Results of experimental studies in experimental animals, in which a strengthened cytotoxicity is observed, have been published. The modification of the anti-tumour response of natural-immunity mechanisms remains one of the most promising ways of treating tumours. But many more functional studies are still necessary in order to determine the role of each immunocompetent cell population in ovarian cancer.

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Comparative study of various subpopulations of cytotoxic cells in blood and ascites from patients with ovarian carcinoma

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