THE ROLE OF IMMUNOLOGICAL MEMORY IN ESTABLISHING ANTITUMOR IMMUNITY IN PATIENTS WITH OVARIAN CANCER UNDERGOING NEOADJUVANT THERAPY

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Understanding the role of immunological memory mediated by T-lymphocytes in patients with malignant tumors is a pressing issue. This study aimed to assess the contribution of memory T-cells to antitumor immunity in patients with ovarian cancer undergoing neoadjuvant chemoimmunotherapy with recombinant interferon-gamma (rIFNγ). Quantification of central (Tcm) and effector (Tem) memory T-cells (Tm), as well as naive T-lymphocytes (Th0), was done using flow cytometry. Compared to healthy females, untreated cancer patients were found to have more Tm and less Th0 cells in their blood $CD4^+$ and $CD8^+$ T-cell subpopulations. In cancer patients, Tm cells accumulated in the ascitic fluid, exceeding 7.7 times the number of $CD4^+$ Th0 cells and 6.5 times the number of $CD8^+$ Th0 cells, with Tem prevailing over Tcm. After chemotherapy with rIFNγ, blood Th0 decreased in cancer patients, while Tcm dominated the $CD8^+$ Tm subpopulation both in the blood and ascitic fluid. Tem cells were a prevalent cell type in patients who received chemotherapy without interferon-gamma. Decreased Th0 and Tcm prevalence were a positive sign accompanied by a good response to treatment, including lower relapse rates (46.7 % vs. 80 % in controls) and a longer relapse-free period (17.5 ± 1.6 vs. 11.3 ± 1.5 months in controls). Therefore, we conclude that chemoimmunotherapy alters proportions of T-cell subpopulations in the blood and ascitic fluid of patients with ovarian cancer, with Tcm cells prevailing over Tem, which may be one of the mechanisms of rIFNγ (Ingaron) action.

Keywords: ovarian cancer, ascitic fluid, blood, flow cytometry, lympocytes, memory T-cells, Tcm, Tem, Th0, interferon-gamma

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РОЛЬ ИММУНОЛОГИЧЕСКОЙ ПАМЯТИ В ФОРМИРОВАНИИ ПРОТИВООПУХОЛЕВОГО ИММУНИТЕТА У БОЛЬНЫХ РАКОМ ЯИЧНИКОВ НА ЭТАПЕ НЕОАДЪЮВАНТНОГО ЛЕЧЕНИЯ

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Изучение роли иммунологической памяти, опосредованной Т-лимфоцитами, при злокачественных опухолях и их лечении — актуальная научная задача. Целью исследования была оценка роли Т-клеток иммунологической памяти в формировании противоопухолевого иммунитета у больных раком яичников на этапе неоадъювантного лечения, включающего химиоиммунотерапию с применением рекомбинантного интерферона-гамма (рИФНγ). Методы: проточная цитометрия для определения центральных (Tcm) и эффекторных (Tem) Т-клеток памяти (Tm) и наивных Т-лимфоцитов (Th0). Результаты: в крови больных по сравнению с донорами обнаружено высокое (Tm) и низкое (Th0) содержание Т-клеток среди $CD4^+$ и $CD8^+$ субпопуляций. В асцитической жидкости происходило накопление Tm, превышающих количество Th0 в 7,7 раза в субпопуляции $CD4^+$ и в 6,5 раза в субпопуляции $CD8^+$, в которой Tem преобладали над Tcm. После химиотерапии с препаратом рИФНγ в крови больных выявлено снижение уровня Th0, в крови и в асцитической жидкости — преобладание Tcm среди $CD8^+$ Tm, а у больных, получавших химиотерапию без иммунотерапии, преобладали Tem. Данные различия были расценены как благоприятные, поскольку сопровождались положительной клинической динамикой: меньшей частотой рецидивирования (46,7 против 80 % в контроле) и более длительным безрецидивным периодом (17,5 ± 1,6 против 11,3 ± 1,5 мес. в контроле). Таким образом, химиоиммунотерапия вызывает перераспределение субпопуляций Т-клеток в крови и в асцитической жидкости при раке яичников в сторону преобладания Tcm над Tem, что может быть одним из механизмов действия препарата «Ингарон» (рИФНγ).

Ключевые слова: рак яичников, асцитическая жидкость, кровь, проточная цитометрия, лимфоциты, Т-клетки памяти, Tcm, Tem, наивные Т-лимфоциты, интерферон-γ

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Rapid development of cell technologies promises advancements in antitumour treatment methods. One of those methods is adoptive immunotherapy that implies application of ex vivo activated lymphocytes of peripheral blood and tumor microenvironment lymphocytes (LAK- and TIL-cells), CIK-lymphocytes, DC-vaccination, etc. [1]. In recent years, a number of studies highlighted efficiency of CAR therapy against certain types of tumors [2].

Nevertheless, adoptive immunotherapy in cancer patients often fails to deliver on expectations, which leads to clinicians abandoning the approach both as alternative to chemotherapy or as a complement to it [1]. Such anti-cancer strategies often fail because of immunosuppressive mechanisms active in cancer patients' bodies, the mechanisms that cause imbalance and dysfunction in various subpopulations of lymphocytes, including lymphocytes powering immunological memory. Immunological memory is not a newly discovered phenomenon. It belongs to the adaptive immunity system that includes T- and B-cell links. Memory lymphocytes play an important part in opposing any chronic pathology. As for tumors, of special importance is the immunological memory mediated by T-lymphocytes. In such cases, researchers pay close attention to $CD8^+$ cells, since, compared to their naive counterparts, those memory cells show a much higher incidence of specific cytotoxic T-lymphocytes recognizing tumor-associated antigens [3]. As the example of melanoma melan-A tetramer shows, in cancer patients, naive T-lymphocytes (Th0) and memory T-cells (Tm) respond ex vivo stimulation with tumor antigen differently; $CD45RA^+CCR7^+$ cells do respond and $CD45RO^+CCR7^-$ do not [4, 5]. Some authors believe the level of $CD8^+$ memory cells in a colorectal cancer tumor and other solid tumors is a key factor for survival of patients [6].

Adoption of lymphocytes immunophenotyping methods in the late 20th century lead to discovery of heterogeneity of Tm lymphocytes subpopulation; as is known now, its subdivision depends on the expression of adhesive, chemokine, costimulatory receptors, on the ability to produce cytokines and the response to them, the ability to penetrate through endothelium and other characteristics. These expressions, abilities and characteristics allowed determining basic subpopulations of Tm: central (Tcm) and effector (Tem). Characteristic for the phenotype of these cells is the CD45RO expression and no CD45RA expression, as well as presence of CCR7, CD62L, CD27 and CD28 on Tcm receptors. As for the Tem cells, they have no CCR7, CD62L and CD28 expression and do show that for CD27 [7]. Th0 boast high CCR7, CD62L, CD27, CD28 and CD45RA expression and no CD45RO. Thus, immunophenotyping with a minimal panel of monoclonal antibodies CD62L, CD45RA and CD45RO allows associating T-lymphocyte to a Th0, Tcm or Tem subpopulation.

Chronic antigenic stimulation alters Th0, which results in not just the aforementioned change in their immunophenotype but also in suppression of proliferation and decreased survival rate, as well weaker response to homeostatic cytokines (IL-7, IL-15) and ability to produce IL-2. At the same time, effector functions, cytotoxicity in particular, grow. CD62L and CCR7 receptors expressed by Th0 and Tcm boost their extravasation through high endothelial venules and migration of peripheral lymph nodes to T-dependent zones, while the Tem inhabit peripheral tissues (liver, lungs) and area of inflammation/tumor [8, 9]. These properties make Tem "watchdogs" and Tcm — "guardians" on the systemic level that ensure a quick response to subsequent antigen administration [10, 11].

As for the antitumor activity of effector and central Tm, there are different opinions, but Tcm is a more popular choice [7, 9]. There is evidence proving that, although they act differently, both types of Tm cells make the antitumor protection optimal, i. e. they complement each other [12]. A number of studies states that if there are $CD4^+$ lymphocytes among Tm, they prevent depletion of $CD8^+$ lymphocytes [13]. Thus, it is better to use a heterogeneous mixture of those subsets [12, 13].

In addition to the basic Tm subpopulations, some researchers point to a resident subpopulation (Trm) formed by circulating $CD8^+$ Tm, which expresses additional CD103 receptors in the nidus [14]. Various phenotypic characteristics of $CD8^+$ Tm in different organs have also been reported. For example, peritoneal cavity normally contains only $CD4^+$ and $CD8^+$ effector lymphocytes, but in case of inflammation $CD4^+$ and $CD8^+$ Tm are also found there [14]. Retention of Tm in the microenvironment with their subsequent transformation into Tm-cells can be triggered by appearance of an antigen [15] or the "cytokine explosion" that accompanies inflammation. Earlier, researchers have described Trm cells' activity in the presence of an inflammation [16, 17], but not in tumor growth which might be a different matter.

There is a number of current research papers covering immunological aspects of ovarian cancer treatment; they are part of effort to develop new immunotherapy methods [1, 18]. Such research often leads to some unexpected findings pertaining to immunological memory cells. For example, one study describes a minor subpopulation of Tm expressing B-cell receptor $CD20^+$ together with $IFN\gamma^+$ and $CD8^+$ [19], which allows assuming its tumor growth suppressing qualities.

Normally, Tcm cells dominate in $CD4^+$ subpopulation; the same is true for Tem in $CD8^+$ subpopulation [11]. The numbers of these cells are different in lymphoid and non-lymphoid organs, and their phenotypic and functional characteristics may differ depending on microenvironment [14].

The immune response to a tumor is both system-wide and local, it activates various cell-based and cell-related factors and also their soluble products [18]. However, it is believed that immunocompetent cells and cytokines present in the tumor's microenvironment can both contribute to this tumor's regression and stimulate its growth, especially at advanced stages [20–23].

Treatment of ovarian cancer (OC) remains one of the biggest challenges for oncologists [24]. According to the available data, OC is the fifth most common cancer. Despite the continuous improvement of diagnostic and treatment methods, it still is the deadliest one. Although chemotherapy is quite efficient against OC, the tumor rarely disappears completely and is prone to recur frequently and early. Today, a combination of surgery and chemotherapy is a standard approach to treating OC at III–IV stages. However, currently it is impossible to make full use of cytoreductive operation due to technical difficulties associated with the spread of tumor. In case surgery is not possible, the first stage of treatment is neoadjuvant chemotherapy, and after the surgery comes adjuvant chemotherapy. The most popular combination for a chemotherapy course is Paclitaxel and Carboplatinum with a 3-week interval [25].

Ingaron, recombinant interferon-gamma (rIFNγ) injectable preparation developed by Russian scientists, aims to stimulate cellular immunity and produces antiviral, antiproliferative and immunomodulating effects [26]. Its antitumor activity derives from the ability to activate natural killers (NK cells), cytotoxic T-lymphocytes and macrophages. Given together with cytostatics, Ingaron helps to decrease the resistance of tumor cells to chemotherapy and thus to make the treatment significantly more effective [27]. Research showed Ingaron to be an efficient medication against cervical cancer, breast cancer,

lung cancer, melanoma, colorectal cancer. The preparation worked even when the stages were advanced [28].

This study aimed to assess the contribution of memory T-cells to antitumor immunity in patients with ovarian cancer undergoing neoadjuvant chemoimmunotherapy with recombinant interferon-gamma (rIFNγ)

METHODS

The study was conducted from 2013 to 2017 at the Department of Gynecologic Oncology, and Laboratory for Immunophenotyping of Tumors, Rostov Research Institute of Oncology (RRIO), Rostov-on-Don, Russia. 30 OC patients aged 34 to 77 underwent examination; the mean age was 54.9 ± 1.3 years. All patients had their tumors detected for the first time. The average duration of a case history was 7 months. All patients voluntarily signed informed consent forms, all examinations followed ethical principles set by the Declaration of Helsinki (2013). Ethical committee of RRIO approved the study by the protocol no. 24 of November 23, 2012.

The inclusion criteria were: 18 years old or older; ascitic form, III–IV stage OC verified cytologically or morphologically; no previous special treatment courses.

The exclusion criteria were: expressed comorbidity (previous myocardial infarction, decompensated heart disease, diabetes mellitus); chemotherapy, radiation therapy, surgery, immunotherapy courses underwent before treatment in the context of the study; metastatic lesions in the central nervous system; pregnancy and lactation; any reasons preventing regular treatment and monitoring sessions.

The patients were divided into 2 groups, 15 patients in each. For group 1 (treatment group) chemotherapy was combined with rIFNγ immunotherapy (ChIT), group 2 (control group) got chemotherapy (ChT) only.

Comparative analysis of the groups revealed that both had 11 (73.3 %) stage III OC patients and 4 (26.7 %) stage IV OC patients. The differences were statistically insignificant ($p > 0.05$). The majority of patients were aged 50–59 years: 5 (33.3 %) in the treatment, 6 (40.0 %) in the control group. No statistically significant difference detected. ECOG-WHO scale was applied to assess the general condition of patients; most scored 2 points (8 (53.3 %) and 9 (60.0 %) patients in treatment group and control group, respectively). The differences were not statistically significant. Thus, age, OC stages and general condition were identical for both groups, which allows making the comparative analysis.

The comparison group consisted of 20 practically healthy women of the same age who had blood samples taken.

The patients were examined as their treatment progressed, before and 3 weeks after ChT (15 patients) and ChIT (15 patients). The 3-week interval matched that between treatment courses. The patients received two to three courses of polychemotherapy: Carboplatinum (AUC-6), intravenously (dropping), Paclitaxel 175 mg/m^2 intravenously (dropping), 21 days between the courses. ChIT regimen was as follows: rIFNγ (Ingaron by Pharmaclone, Russia) injected intramuscularly, 500,000 IU on day 1, 1,000,000 IU on days 2, 3 and 5, and day 4 was for Paclitaxel + Carboplatinum (polychemotherapy). Age, OC stages and general conditions were identical for both groups.

Further, all patients underwent surgery depending on the results of the treatment. It could be total hysterectomy with salpingo-oophorectomy, omentum resection or extirpation, salpingo-oophorectomy. Post-operation, treatment group patients received adjuvant ChIT: Ingaron, intramuscularly, same regimen started on the 9th day. The patients had 2 Ingaron cycles and then 4 chemotherapy cycles, which made the entire adjuvant treatment course included 6 cycles. Control group patients received 6 ChT cycles.

Complete or partial regression and the general effect (tumor regression and stabilization) and progression values as defined by WHO were criteria for assessment of the effect produced by neoadjuvant therapy.

The post-treatment follow-up period was 3 years, with tumor recurrence time registered.

Blood and ascitic fluid (AF) samples were taken before the treatment and at its stages. The total number of blood samples examined was 98, number of AF samples equaled 47.

Blood lymphocytes and AF flow cytofluorimetry. The device used for the purpose was BD FACSCanto II flow cytometer (Becton Dickinson, USA). It features two lasers emitting at 488 nm and 633 nm (fluorophores excitation wavelengths) and allows using up to 6 monoclonal antibodies in one tube simultaneously. Monoclonal antibodies were conjugated with the following fluorochromes: FITC, PE, PerCP-Cy5.5, PE-Cy7, APC, APC-Cy7. The cytometer was set up with the help of standard BD FACS 7-color setup beads (BD Biosciences, USA).

Sample preparation and immunophenotypic staining. To assess expression of receptors, researchers resorted to immunophenotypic staining. It was done with fluorescently-labeled antibodies as prescribed by the protocol supplied by the manufacturer. For each examination, 2 tubes with the following sets of monoclonal antibodies BD Multitest (Becton Dickinson, USA) were used:

1. CD45RA FITC/CD45RO PE/CD3 PerCP/CD4 APC (cat. #340571) or CD45RA FITC/CD45RO PE/CD3 PerCP/CD8 APC (cat. #340574);
2. CD45RA FITC/CD62L PE/CD3 PerCP/CD4 APC (cat. #340977).

This panel of antibodies allows to count the content of Th0, Tcm and Tem ($CD62L^+CD45RA^+CD45RO^-$, $CD62L^+CD45RA^-CD45RO^+$ and $CD62L^-CD45RA^-CD45RO^+$ respectively) among $CD4^+$ and $CD8^+$ Tm subpopulations. At least 50,000 cells were accumulated in each sample for data analysis.

Gating tactics and data analysis. The lymphocyte region was determined with the help of Dot Plot chart by direct (relative cell size) and lateral (cell structure) light scattering parameters. The share of T-helpers and cytotoxic T-lymphocytes in the overall lymphocytes population was calculated within this range by markers CD3, CD4 and CD8. Next, we analyzed the share of T-lymphocytes with phenotypes $CD4^+CD45RO^+CD45RA^-$, $CD8^+CD45RO^+CD45RA^-$, $CD4^+CD45RA^+CD62L^+$, $CD8^+CD45RA^+CD62L^+$ in the populations of T-helpers and cytotoxic T-lymphocytes. For each sample, Tm/Th0 coefficients were calculated for $CD4^+$ and $CD8^+$ subpopulations. Tcm and Tem levels were taken as a percentage of the number of Tm belonging to $CD4^+$ and $CD8^+$ subpopulations, then the Tem/Tcm coefficient was calculated. Fig. 1 and 2 show examples of blood and AF flow cytofluorimetry results.

Mathematical and statistical processing methods. BD FACSDiva Software (Becton Dickinson, USA) did mathematical processing of the data. Statistica 8.0 for Windows and MS Excel were the tools that enabled the results analysis and statistical processing. The threshold of statistical significance of differences was $p < 0.05$. The Wilcoxon test was also used; the χ^2 fitting criterion helped to assess reliability of differences between frequency of development of total and overall effect and frequency of recurrence.

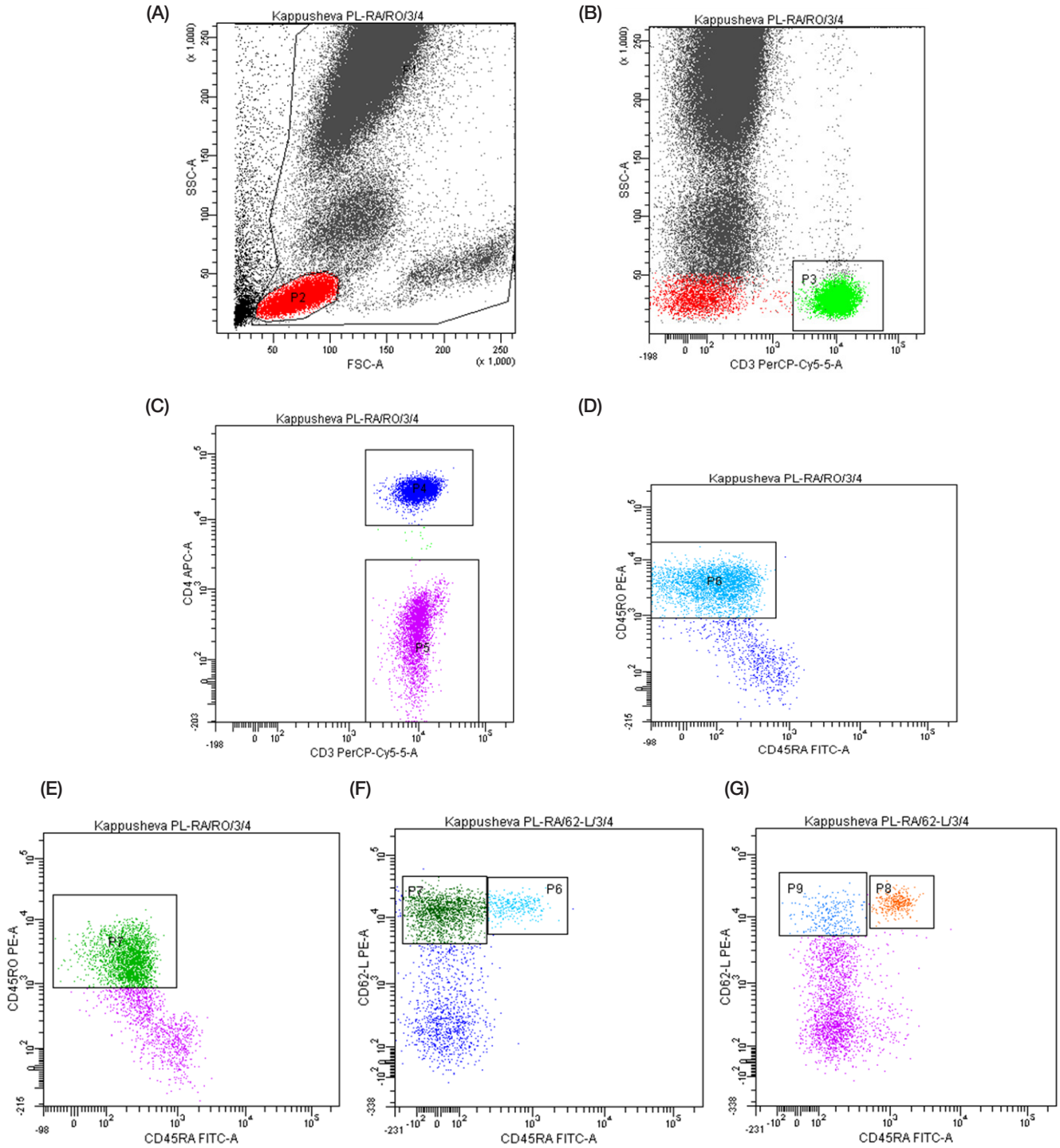

Fig. 1. Peripheral blood Tm, flow cytometry: **(A)** — lymphocyte region among blood cells; **(B)** — T-lymphocytes population; **(C)** — distribution of CD4 and CD8; **(D)** — distribution of CD45RA and CD45RO after logical restriction by CD4; **(E)** — distribution of CD45RA and CD45RO after logical restriction by CD8; **(F)** — distribution of CD45RA and CD62L after logical restriction by CD4; **(G)** — distribution of CD45RA and CD62L after logical restriction by CD8

RESULTS

Tables 1–4 and in Fig. 3 and 4 show the results of the study. We found some differences in content of Tm and Th0 lymphocytes in biological fluids samples. As seen in Table 1, blood of OC patients has a higher level of Tm among $CD3^+CD4^+$ cells and a lower level of Th0 among $CD3^+CD8^+$ cells compared to those seen in healthy women; the difference is statistically significant ($p < 0.05$). Comparison of levels of Tm in blood and AF of OC patients revealed that these cells are much more common in AF ($p < 0.05$). In contrast, the levels of Th0 in AF were statistically significantly lower when compared to those seen in the patients's blood ($p < 0.05$) (Table 1).

There were no statistically significant differences between $CD4^+$ and $CD8^+$ cell levels in blood and AF, although levels of lymphocytes and monocytes in AF significantly exceeded those seen in blood. Despite the significantly higher level of lymphocytes in AF (52.4 ± 5.5 compared to 17.4 ± 2.7 % in patients' blood), the contents of their main subpopulations in these biological fluids were not statistically different: the level of $CD4^+$ cells in blood was 49.2 ± 3.1 %, that in the AF — 48.7 ± 3.2 %; $CD8^+$ levels were 18.4 ± 2.7 and 22.6 ± 3.3 %,

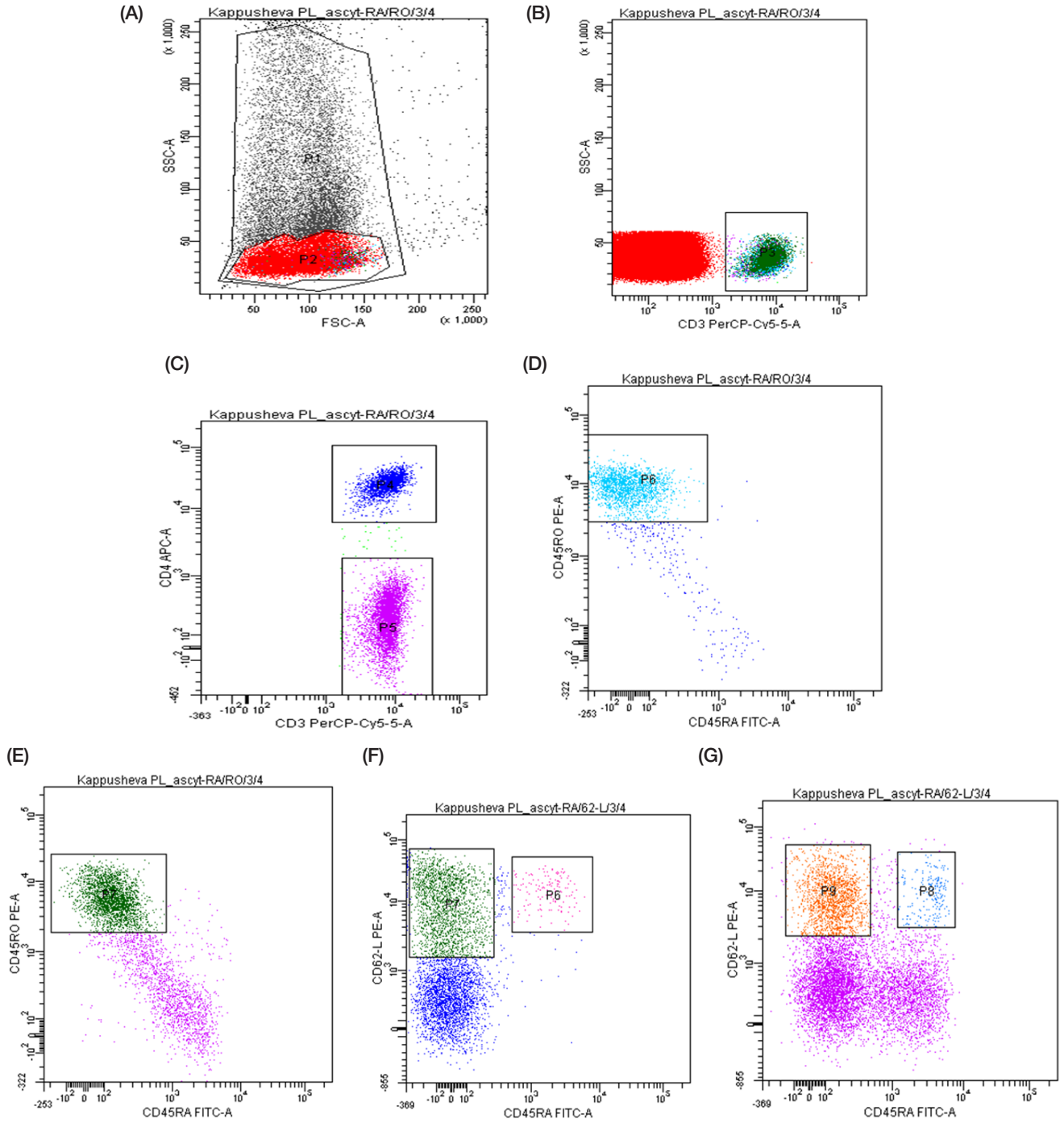

Fig. 2. AF Tm, flow cytometry: **(A)** — lymphocytes region; **(B)** — T-lymphocytes population; **(C)** — distribution of CD4 and CD8; **(D)** — distribution of CD45RA and CD45RO after logical restriction by CD4; **(E)** — distribution of CD45RA and CD45RO after logical restriction by CD8; **(F)** — the distribution of CD45RA and CD62L after logical restriction by CD4; **(G)** — distribution of CD45RA and CD62L after logical restriction by CD8

respectively. The $CD4^+/CD8^+$ index for AF was also on par with that for blood of patients, i. e. there were no statistically significant differences there. However, the ratio of Tm/Th0 found in both T-cell subpopulations was significantly higher in AF of patients than in their blood (7.7 ± 1.0 vs. 2.9 ± 0.5 for $CD4^+$ cells and 6.5 ± 1.3 vs. 2.1 ± 0.3 for $CD8^+$ cells; in both cases, $p < 0.05$). This means there are markedly more Tm than Th0 in the tumor nidus than in the peripheral blood.

We also found some differences in content of Tm subpopulations in the examined biological fluids (Table 2).

Table 2 shows that Tem levels among subpopulations of $CD4^+$ and $CD8^+$ cells in AF are statistically significantly higher than those in blood of patients and in blood of healthy women ($p < 0.05$). As for the content of Tcm, the differences between AF of patients and blood of healthy women were found for $CD8^+$ only. It is interesting that there were no statistically significant differences in the studied subpopulations in blood of patients and healthy individuals.

Tem levels in $CD4^+$ and $CD8^+$ subpopulations were similar in most cases, whereas the share of Tm in $CD4^+$ subpopulation was 2–2.3 times higher than in $CD8^+$ subpopulation. In blood and AF samples, Tem dominated over Tcm in $CD8^+$ cell subpopulation (over 1.0), and Tcm dominated over Tem in $CD4^+$ subpopulation (below 1.0) (Fig. 3).

Thus, comparison of systemic and local cellular immunity indices seen in AF of OC patients showed that Tm dominate in

patients' blood when put against that of healthy women and in the patients' AF when compared to their peripheral blood. Most of the $CD4^+$ Tm were Tcm while the majority of $CD8^+$ Tm were Tem, which accumulated in patients' AF.

Neoadjuvant treatment lead to a complete regression of tumor in 4 patients (26.7 %) of the treatment group (ChT plus rIFNγ) and in 1 (6.7 %) belonging to the control group. Assessment of the overall effect of treatment revealed statistically significant differences in data describing treatment group and control group (87.0 and 30.0 %, respectively). Only 1 patient of the control group had the tumor progressing (6.7 %).

After 2–3 cycles of neoadjuvant polychemotherapy, 12 (80.0 %) patients of the treatment group underwent full-scale surgery (total hysterectomy with salpingo-oophorectomy, omentum resection or extirpation). In control group, only 6 patients were in a state allowing this degree of intervention. The difference is significant ($p < 0.05$). 3 patients (20.0 %) of the treatment group had incomplete surgery that included oothecoma removal and omentum resection; in the control group, the same operations were performed on 8 patients (53.3 %), and 1 patient had a trial laparotomy.

The post-adjuvant treatment follow-up period was 3 years. Recurrence in the treatment group was 46.7 % while that in the control group was 80.0 % (7 and 12 patients, respectively). The average time of the recurrence was 17.5 ± 1.6 months and 11.3 ± 1.5 months, respectively. The differences are significant ($p < 0.05$).

Only the patients that had the same kind of surgery (full-scale surgery) were picked for immunological examination.

Dynamics of levels of Tm and Th0 in blood of patients from both groups can be seen in Table 3. There are some differences there. Worth a special note is the decrease of levels of naive Th0 in treatment group patients (ChT plus rIFNγ), which became statistically significant by the end of the treatment ($p < 0.05$). In the control group, the level of these cells did not differ from the initial one during the entire follow-up period.

The Tm/Th0 ratio was growing (and reached statistical significance) in the treatment group and not in the control group (Fig. 4). Thus, the $CD8^+$ cells Tm/Th0 ratio increased from 2.5 ± 0.5 to 3.4 ± 0.7 in patients of the treatment group, while in the control group it went down from 1.7 ± 0.3 to 1.4 ± 0.3. The difference is significant ($p < 0.05$). As for the $CD4^+$ subpopulation, the growth was 2.5-fold in the treatment group and in the control group it did not change.

During treatment, Tm content was not significantly different in treatment and control groups (Table 3), but assessment of dynamics revealed such differences in $CD8^+$ Tm subpopulation (Table 4). In the control group, the level of Tcm gradually decreased and by the end of the treatment the difference acquired statistical significance ($p < 0.05$). Tem levels did not change.

ChIT that included rIFNγ initially led to similar changes, but as the treatment approached completion, the volume of Tcm was greater and Tem lower in blood of treatment group patients that those in blood of control group patients (Table 4). In other words, Tcm dominated in blood of the treatment group patients and Tem were the majority in the control group patients' blood. The Tem / Tcm ratio was 2.27 ± 0.4 and 0.62 ± 0.18, respectively, $p < 0.05$.

The dynamics of levels of the studied subpopulations in AF could only be registered when the fluid accumulated, i. e. during the preoperative period. It was found that the Tm/Th0 levels ratio in $CD4^+$ lymphocytes subpopulation increased from 7.7 ± 1.0 to 13.0 ± 1.5 ($p < 0.05$); the data describes treatment group, the increase is statistically significant. AF of patients of the same group has also shown changes in Tm $CD8^+$ subpopulation: the level of Tem decreased (13.6 ± 4.6 compared to 42.1 ± 4.1 % in the control group, $p < 0.05$), and the volume of Tcm tended to increase. The total number of lymphocytes in AF after ChIT was 42.2 ± 7.8, while after ChT it was 31.1 ± 7.1 % (the differences are statistically insignificant); However, ChT lead to the reduction in their numbers compared to the initial counts, the difference here being statistically significant (52.4 ± 5.5 %, $p < 0.05$). Other statistically significant differences include those describing $CD3^+$ and $CD4^+$ cell levels: ChIT brought them up to 82.1 ± 6.3 and 57.6 ± 5.7, respectively, while ChT produced a more modest growth of 65.4 ± 6.5 % and 42.6 ± 2.5 %, respectively; in both cases $p < 0.05$.

Thus, the content of Tm in both $CD4^+$ and $CD8^+$ subpopulations in patients' AF was greater than that in their blood, while Th0 was smaller; among $TmCD8^+$, Tem were prevalent. The study revealed that adding Ingaron (rIFNγ) to a chemotherapy course, thus making it a chemoimmunotherapy

Table 1. Tm and Th0 subpopulations of $CD4^+$ and $CD8^+$ in blood and AF of OC patients

Samples	Tm, %		Th0, %	
	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
Blood of healthy women (n = 20)	55.0 ± 3.7	35.0 ± 4.3	23.4 ± 3.2	27.7 ± 3.7
Blood of OC patients (n = 30)	64.5 ± 2.3*	40.8 ± 5.1	22.4 ± 4.1	18.5 ± 2.7*
AF of OC patients (n = 30)	79.2 ± 4.0*,**	57.5 ± 3.1*,**	10.2 ± 1.6*,**	9.5 ± 2.5*,**

Note. * — significant differences from blood of healthy donors ($p < 0.05$); ** — significant differences from blood of patients ($p < 0.05$). Hereinafter, in tables 2–4 reliability of the differences was calculated through the Wilcoxon test.

Table 2. Central and effector Tm with $CD4^+$ and $CD8^+$ phenotype in blood and AF of OC patients

Samples	Tm $CD4^+$		Tm $CD8^+$	
	Tcm, %	Tem, %	Tcm, %	Tem, %
Blood of healthy women (n = 20)	41.7 ± 2.5	17.8 ± 2.3*	17.7 ± 2.5*	24.1 ± 3.6*
Blood of OC patients (n = 30)	42.0 ± 3.1	24.8 ± 1.8*	22.0 ± 1.5	28.2 ± 3.2*
AF of OC patients (n = 30)	47.1 ± 3.3	39.8 ± 3.9	27.0 ± 2.0	42.1 ± 4.1

Note. * — significant differences from AF ($p < 0.05$).

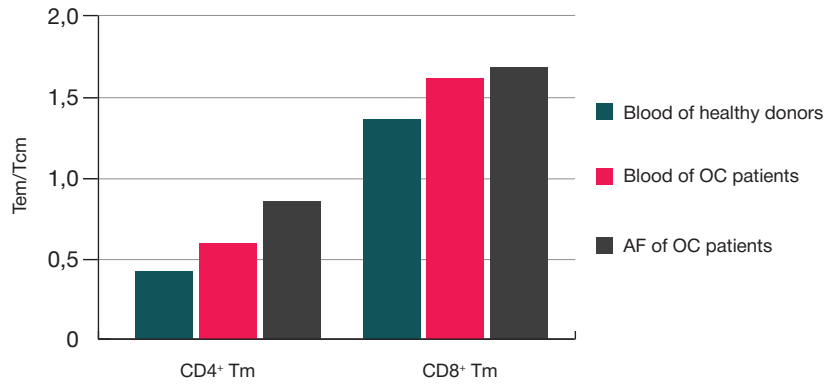

Fig. 3. Tem / Tcm ratio in CD4+ and CD8+ subpopulations of memory T-cells

course, maintains the opposite dynamics of Tem and Tcm cells in patients' blood and AF.

DISCUSSION

The biological role Tm play in resisting cancer is important. The volumes and ratios of these cells can be changed through immunotherapy. Within the context of this study, we monitored and registered the levels of Tm (including Tcm, Tem) and Th0 in blood and ascitic fluid (AF) of OC patients before treatment and during chemoimmunotherapy. Earlier, we registered and described a higher content of lymphocytes (with Tm dominating) in AF of patients [29]; this study goes further and discovers the prevalence of Tem there, mostly belonging to CD8+ subpopulation. Although it is not yet possible to establish their specificity and substantiate the hypothesis of their antitumor activity, the published research data available indicate that, since the expansion of lymphocytes as part of a lymphoid infiltrate in the tumor (tumor-infiltrating lymphocytes) is clonal, antigen-specific lymphocytes should dominate in the tumor microenvironment [6]. Judging by the noticeable spread of tumor, OC patients do not develop effective immune reactions, which may be caused by some peculiar features of the tumor microenvironment. It appears that tumor cells in AF activate peritoneal macrophages and monocytes, which leads to hyperproduction of cytokines possessing pro-tumor properties [21]. AF monocytes are a potential source of type 2 macrophages that support tumor growth by autocrine production of VEGF, EGF, TGFß, IL-6, IL-8, IL-10, supported by hypoxia developing in tumor tissue [23, 30]. These same cytokines, as well as chemokines that can be produced by tumor cells, stimulate the migration of lymphocytes into the peritoneal cavity. According to the published data, there are natural T-regulatory cells (T-regs) among them, and they suppress immunity and boost tumor growth. OC patients have more such T-regs than healthy women [31], and the cells are much more abundant in patients' AF than in their blood [32]. They probably block the activity of T-lymphocytes, Tm in particular and other lymphocytes (NK-, CD8+) in general. Those lymphocytes, although abundant, seem to be hindered in their functions, as shown by the example of CD8+ Tm chronically stimulated in the presence of viral infections [7, 9, 33, 34]. Unlike T-regs, Tm die on schedule (apoptosis) when interacting with FasL expressed by endotheliocytes of tumor vessels; this may be one of the self-protective mechanisms the tumor has [35]. Perhaps, some measures countering local immunosuppression could allow effective inclusion of cellular factors into antitumor immune response that would rise the effectiveness of OC treatment.

As we have reported earlier, Ingaron added to a chemotherapy course causes a decrease in the level of Th0 in OC patients and otherwise positively affects their immunity [36]. In this study, we focused on learning more about Tm in OC patients. We have found that introduction of rIFNγ ensures domination of Tm over Th0 in blood and AF of OC patients after chemoimmunotherapy. It also provokes redistribution of Tm CD8+ and brings around more Tcm, which is not seen in patients that receive chemotherapy without rIFNγ. In part, this may be the reason behind the improved clinical effect observed when rIFNγ joins chemotherapy. Although the reports published to date indicate that repeated administration of the antigen provokes proliferation and increases cytotoxicity both of Tcm and Tem (part of CD8+ T-cell subpopulation), it is believed that Tcm has these properties more pronounced and also shows a more active interaction with antigen-presenting cells, higher antigen-induced proliferation, generation of cytotoxic T-effectors and homing into secondary lymphoid organs [7, 37]. This suggests the advantage tumor-specific CD8+ Tcm have when compared to Tem as antitumor factors. Thus, the data we have received can be interpreted as one of the therapeutic mechanisms Ingaron offers against ascitic forms of OC.

Table 3. Dynamics of Tm and Th0 levels, blood of OC patients, treatment and control groups

Group of patients	Treatment term	Tm, %		Th0, %	
		CD4+	CD8+	CD4+	CD8+
Treatment (n = 12)	before treatment	65.8 ± 4.2	40.9 ± 5.2	20.5 ± 3.5*	17.2 ± 1.6*
	after 2–3 ChIT cycles	64.6 ± 6.1	37.7 ± 4.5	15.7 ± 3.4	16.5 ± 2.3
	after full ChIT course	72.8 ± 5.9	35.7 ± 5.1	9.7 ± 2.7	10.4 ± 2.6
control (n = 6)	before treatment	66.3 ± 4.4	38.1 ± 4.7	23.6 ± 4.6	23.6 ± 5.6
	after 2–3 ChT cycles	59.1 ± 7.1	31.6 ± 4.5	16.8 ± 3.9	18.8 ± 3.2
	after full ChT course	63.2 ± 7.3	37.5 ± 7.2	24.4 ± 6.2*	27.3 ± 7.5*

Note. * — significant differences from the parameters of the treatment group after full ChIT course ($p < 0.05$).

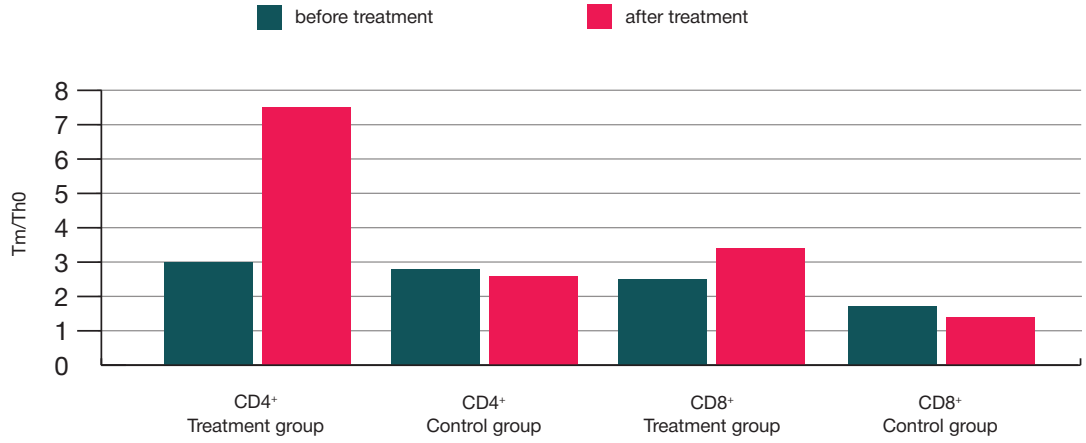

Fig. 4. Changes in Tm / Th0 ratio, CD4+ and CD8+ subpopulations, blood of OC patients before and after treatment

Table 4. Dynamics of Tcm and Tem levels, blood of OC patients, treatment and control groups

Group of patients	Treatment term	Tm CD4+		Tm CD8+	
		Tcm, %	Tem, %	Tcm, %	Tem, %
Treatment (n = 12)	before treatment	43.0 ± 2.5	26.3 ± 3.8	24.4 ± 3.6	25.8 ± 3.3
	after 2–3 ChIT cycles	39.8 ± 4.1	26.8 ± 3.3	13.3 ± 3.4*	22.6 ± 3.7
	after full ChIT course	42.8 ± 3.8	28.4 ± 5.1	22.2 ± 2.9**	13.9 ± 3.6*,**
Control (n = 6)	before treatment	42.3 ± 3.6	25.5 ± 2.8	22.5 ± 2.3	30.6 ± 4.9
	after 2–3 ChT cycles	45.3 ± 4.7	23.7 ± 3.7	17.8 ± 4.0	25.3 ± 2.9
	after full ChT course	43.1 ± 6.1	23.8 ± 4.7	12.6 ± 2.2*	28.7 ± 3.3

Note. * — significant differences from values seen before treatment ($p < 0.05$); ** — significant differences from the corresponding values of the control group ($p < 0.05$).

CONCLUSIONS

Before treatment, OC patients (ascitic forms) have Tcm dominating in CD4+ subpopulation of Tm and Tem dominating in CD8+ subpopulation of Tm, which accumulate in ascitic fluid (AF). Despite the high levels of Tm in blood and AF, their function seems to be injured and therefore it is important to study the ways to correct it.

Compared to patients that received plain chemotherapy, those who had chemoimmunotherapy course with recombinant interferon-gamma showed better clinical effects: decreased volume of Th0 in blood, domination of Tcm over Tem in CD8+ Tm subpopulation. Since the like changes take place in AF too, such modulation of the tumor's immunological microenvironment can boost the clinical effect and, in particular, ensure longer recurrence-free period in OC patients.

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