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Analysis of the Treg cell population in the peripheral blood of ovarian cancer patients in relation to the long-term outcomes

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ABSTRACT

Objectives: There is growing evidence that Treg cell infiltration into the cancer nest is associated with poor prognosis. However, the Treg cell population in the peripheral blood may change when a different type of anticancer therapy is applied. Since Treg cells may support tumor growth by enhancing the suppressive profile of the cancer microenvironment, the assessment of Treg cells can bring to light important information regarding prognosis. Thus we decided to analyze the Treg cell population in the peripheral blood in relation to long-term outcomes in the group of patients with ovarian cancer.

Material and methods: The 80 patients included in the study were treated surgically followed by chemiotherapy for ovarian cancer between October 2010 through May 2011.The peripheral blood samples from the patients were collected directly prior to chemotherapy. Information on any patients who died was retrieved from the database of the Cuiavia-Pomerania Regional Office of the National Health System of Poland. CD4+CD25+FOXP3+ lymphocytes T were assed by flow cytometry. We have analyzed the long term outcomes of treatment regarding to the level of Treg cells in peripheral blood.

Results: We found that patients with serous adenocarcinomas had significantly higher Treg levels compared to those patients with non-serous types. Patients who had a higher percentage of Treg cells within the CD4+ cell population prior to the beginning of the treatment had worse long-term outcomes from the applied therapy.

Conclusions: The assessment of Treg levels prior to the start of chemotherapy is clinically useful and may predict overall survival in ovarian cancer patients.

Key words: regulatory T-cells; Tregs; ovarian cancer; tumor immunology

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INTRODUCTION

Regulatory T cells (Tregs) represent 1.3% of the total $CD4^+$ cell population [1]. Tregs are distinguished from other T cells by the expression of the transcription factor Forkhead box protein 3 (FoxP3) [2]. The transcription factor FoxP3 is crucial for suppressing the activity of Treg cells; it directly suppresses IL-2 gene transcription and upregulates CTLA-4 and IL-2 receptor α-chain gene transcription [3]. The population of Treg cells consists of naïve (nTreg, FoxP3 low, and CD45RA+) and effector (FoxP3 high and CD45RA) Treg cells

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[4]. These cell scan both suppress the activation of immune system cells and block already activated T lymphocytes. It is well known that Treg cells can suppress most immune cells including CD4+ and CD8+ lymphocytes, B cells, NK cells, NKT cells, APCs, monocytes, macrophages, and DCs [2]. Regulatory T cells (Tregs) are an important contributor to the immunosuppressive tumor microenvironment and, consequently, they play a critical role in the suppression of inflammation and the process of cancer cell evasion of the host immune surveillance [5].

Recently, it has been proven that in patients with some types of cancers (for example, breast [6], lung [7], urinary bladder [8], and ovarian cancers [9, 10] the Treg cell subpopulation size correlates withan advanced stage of cancer. However, there is no such correlation with the histological tumor type, for instance, squamous vs adenocarcinoma in patients with lung cancer [7] and mucinous vs serous adenocarcinoma in patients with ovarian cancer [10]. Wu et al. demonstrated differences in the Treg population size which correlated with whether the tumor was malignant or benign. The level of Treg cells in the peripheral blood was significantly higher in the patients with ovarian cancers compared to those with benign ovarian tumors [10]. Curiel et al. [11] observed that the infiltration of the ovarian cancer nest by Treg cells has a negative impact on prognosis, and Zhou et al. [4] showed that the Il-10 secreted by TAMs increased the number of Treg cells through the activation of FoxP3 during T-cell differentiation and promotes tumor progression. Recently, it has been demonstrated that the monoclonal antibodies against CTLA4 and PLD-1, proteins present on the Treg cell membrane, are clinically useful in therapy for patients with various types of cancers, and both are key components of Treg cell activity [1].

On the other hand, the Treg cell population in the peripheral blood may change when a different type of anticancer therapy is applied. Previous studies have shown that radical cytoreductive surgery in ovarian cancer patients was associated with a significant decline in the Treg population analyzed in the peripheral blood [12]. Wu et al. observed a significant fall in the postoperative level of Treg cells in comparison to the preoperative level. The lowest concentration of Treg cells was observed 30 days after surgery [10]. Chen et al. [7] proved that the level of Treg cells dropped significantly following lung cancer surgery. The application of the radiochemotherapy has also been proven to influence the Treg cell level. Wang et al. demonstrated that in the patients with stages II/III breast cancer the percentage of the Treg cells fell significantly following the six cycles of chemotherapy [6]. Shaobin et al. [13] observed that the radiofrequency ablation in lung cancer patients decreased the percentage of Treg cells within CD4+ lymphocytes in the peripheral blood.

However, cancer treatment is a complex process consisting mostly of classic chemotherapy, radiotherapy, and surgery. Moreover, it has been demonstrated that surgery and chemoradiotherapy influence the Treg cell population and enhance the antitumor immune response. They interrupt the stabilizing pathways of Treg cells, making them more sensitive to the immunotherapy. Further studies on this subject are needed to elucidate the emerging pathways of Treg stabilization and destabilization and could reveal new molecular targets for therapy [14].

Since Treg cells may support tumor growth by enhancing the suppressive profile of the cancer microenvironment, the assessment of Treg cells can bring to light important information regarding prognosis.

There is growing evidence that Treg cell infiltration into the cancer nest is associated with poor prognosis. The findings in the literature on this subject are conflicting. For example, Curiel et al. [11] demonstrated a correlation between the level of Treg cells and both high death hazard and reduced survival in a group of ovarian cancer patients. The patients with the highest Treg cell infiltration had a 25 times greater risk of death compared to the patients with the lowest levels of Treg cell infiltration [11]. Mhawech-Fauceglia et al. reported that in patients with familial ovarian cancer,the size of the Treg cell population was the only significant predictor of prognosis. They also proved that high tumor grade correlates with the infiltration of Treg cells [15]. Contrarily, Leffers et al. analyzed patients with advanced stage ovarian cancer and observed that CD8+ and FoxP4+ Treg infiltration was an independent predictor of improved prognosis. Furthermore, the relationship between Treg cell levels and survival was analyzed in patients with other types of cancers, including breast cancer [16]. By contrast, Noordam et al. found no correlation between the overall Treg level and survival. However, in this study, when the authors distinguished naive from effector Treg cells, they observed that the nTreg level corresponded with improved survival [17]. Noordam et al. [17] have demonstrated that the number of naïve Treg (nTreg) cells in the peripheral blood positively correlates with survival in malignant pleural mesothelioma patients.

Objectives

Thus we decided to analyze the Treg cell population in the peripheral blood in relation to long-term outcomes in the group of patients with ovarian cancer treated by a cytoreductive surgery followed by six courses of a chemotherapy based on platins and taxans.

MATERIAL AND METHODS

Study group

The 80 patients included in the study were treated surgically for ovarian cancer between October 2010 through May

2011 in the the Department of Gynecology and Oncology of the Lukaszczyk Oncological Center, Bydgoszcz, Poland. The median patient age was 58 years (range 35–81). As the average age at which a woman goes through menopause in Poland is 51 years, women in the study over the age of 51 were assessed as postmenopausal. The study included 18 premenopausal and 62 postmenopausal women. All patients underwent standard surgical treatment for ovarian cancer. Tumors removed during surgery were examined histopathologically and classified according to the WHO criteria.

The study group included 53 high-grade serous adenocarcinomas, 8 mucinous adenocarcinomas, 5 clear cell adenocarcinomas, 10 endometrioid adenocarcinomas, and 4 nondifferentiated adenocarcinomas. Thirty-eight tumors were graded as G3, 33 as G2, and 9 as G1 tumors. EOCs were classified according to the then-current International Federation of Gynecology and Obstetrics (FIGO) system. Patients were subdivided according to the FIGO stage of the disease as follows: 14 stage I patients, 10 stage II patients, 53 stage III patients, and 3 patients had disease classified as stage IV.

The study group consisted of 80 samples of the primary EOCs. Blood samples from the patients were collected directly prior to chemotherapy. The samples were collected in a serum collection tube. A clot was allowed to form at a room temperature for 30–60 minutes. Next, the tube was placed on ice for 30 minutes to contract the clot stored at -80°C.

The median follow up for our patients was 1.163 days (range 34–3.142 days). Information on any patients who died was retrieved from the database of the Cuiavia-Pomerania Regional Office of the National Health System of Poland. The patient's consent was obtained in each case. Prior to the present study, we also obtained the approval of the Jagiellonian University Ethical Committee for our research program (DK/KB/CM/0031/447/2010)

Flow cytometry

The samples for the cytometric analysis of the Treg cell population in the whole blood of patients and healthy donors were prepared using Human Regulatory T Cell Staining Kit (eBioscience), according to the manufacturer's instructions with some minor modifications. To the 100 µL of blood, 15 µL of CD4 FITC and CD25 PE cocktail, as well 5 µL of CD45 APC-Cy7 (Becton Dickinson) was added. After 30 min of incubation with mAbs (in the dark at 4°C) the cells were washed with Flow Cytometry Staining Buffer, centrifuged for 5 min at 350 × g, and then permeabilized with freshly prepared Fixation/Permeabilization Buffer (for 40 min in the dark at 4°C). Next, the cells were washed twice by adding 1× Permeabilization Buffer, blocked by normal rat serum (for 15 min in the dark at 4°C) and subsequently stained with anti-human FoxP3 APC antibody or rat IgG2a κ APC antibody (in the case of isotype control) for 35 min in the dark at 4°C. After another washing step, the cells were suspended in Flow Cytometry Staining Buffer and analyzed using BD FACS Canto II flow cytometer and BD FACS Diva Software (Becton Dickinson). In each sample, 3 × 104 lymphocytes were collected and gated on SSC × CD45 APC-Cy7 dot-plot. Next, the populations of CD4+ FITC, CD25+ PE, and double-positive CD4 and CD25 cells were distinguished among the lymphocytes. Finally, the gate of FoxP3 positive cells was established on CD4+CD25+ subpopulation. For each sample, the negative control was performed on the specific-stained cell analysis in order to measure autofluorescence and the isotype control was performed in order to exclude non-specific staining of specific antibodies.

Statistical analysis

The nonparametric Mann-Whitney test was used to compare Treg levels according to menopausal status, FIGO stage, tumor grade, and the histopathological type of the tumor. The correlation with patient age was performed using the non-parametric Spearman Rank Correlation. Survival analyses were conducted using the Kaplan-Meier survival curves. The groups for survival analyses were distinguished according to median Treg levels. A result was considered significant when the P-value was less than 0.05.

RESULTS

The patients with low pre-chemotherapy Treg levels (below median [3.7]) have significantly longer overall survival compared to the patients with high (above median) pre-chemotherapy Treg levels (1.636 days, range 34–3.142 vs 1.001 days 35–2.962; P = 0.024). Figure 1 presents the survival curves. No correlation between Treg levels and patient age was found (R Spearman = -0.001, P = 0.992). Similarly, Treg levels were not

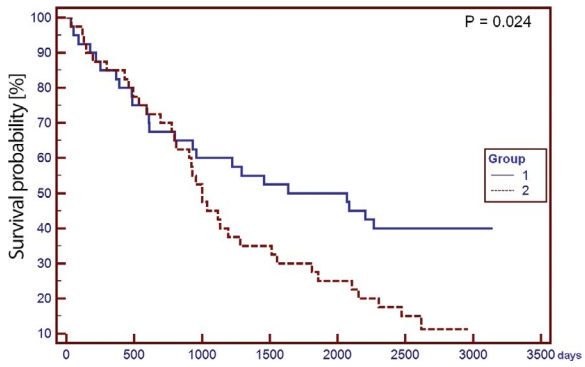

Figure 1. The comparison of the overall survival curves of the patients with low and high pre-chemotherapy Treg levels. Group 1: Patients with low (below median [3.7]) pre-chemotherapy Treg levels (median survival 1.636 days, range 34–3.142). Group 2: Patients with high (above median) pre-chemotherapy Treg levels (median survival 1,001 days, range 35–2.962; P = 0.024).

Table 1. Treg levels according to clinicopathological features of ovarian cancer disease

	Median (range)	P — value
Premenopausal women	3.5 (0.3–7.4)	P = 0.56
Postmenopausal women	3.8 (0.1–18.5)	
	Median (range)	P — value
FIGO I	1.95 (0.2–4.5)	P = 0.076
FIGO II	3.2 (0.7–18.5)	
FIGO III	4.0 (0.1–10.5)	
FIGO IV	5.1 (2.8–7.0)	
	Median (range)	P — value
G1 and G2	2.8 (0.4–18.5)	P = 0.039
G3	4.1 (0.5–9.4)	
	Median (range)	P — value
Serous adenocarcinoma	3.8 (0.1–18.5)	P = 0.033
Others	2.3 (0.3–8.0)	
	Median (range)	P — value
Serous adenocarcinoma	3.8 (0.1–18.5)	P = 0.066
Mucinous adenocarcinoma	1.95 (1.0–4.2)	
Endometrioid adenocarcinoma	2.35 (1.2–4.5)	
Clear-cell adenocarcinomas	0.3 (0.2–1.1)	
Non-differentiated tumors	4.3 (1.1–8.0)	
Non-serous adenocarcinoma	2.3 (0.3–8.0)	

influenced by menopausal status (P = 0.56). Table 1 presents the level of Treg cells in correlation to the FIGO stage of the disease.

We found significantly higher pre-chemotherapy Treg levels in patients with grade 3 tumors, compared to those with G1/2 tumors. Within all of the studied groups, there were no differences in Treg levels in relation to the histopathological type of the tumor. However, when the Treg levels of patients with serous adenocarcinomas and patients with other types of ovarian malignancies were compared, there was a statistically significant difference. Table 1 summarizes the results.

DISCUSSION

We found that patients with serous adenocarcinomas had significantly higher Treg levels compared to those patients with non-serous types. Patients who had a higher percentage of Treg cells within the CD4 + cell population prior to the beginning of the treatment had worse long-term outcomes from the applied therapy.

Many studies [9, 11, 18] have observed the association between Treg cells, poor prognosis, and unfavorable disease characteristics; however, the findings have conflicted. Curiel et al. studied Treg cell levels in ovarian cancer patients, focusing on Treg cell infiltration into the tumor nest, and found a correlation between Treg cells and both reduced survival and high death hazard. Cannioto et al. [9] confirmed the increased frequency of Treg cells inthe peripheral blood of ovarian cancer patients in comparison to patients with benign tumors and to a healthy control group. In a study by Mhawech-Fauceglia et al., high tumor grade correlated with the infiltration of Treg cells. Moreover, the size of the Treg cell infiltration was the only significant positive prognostic predictor in patients with familial ovarian cancer [15]. Leffers et al. analyzed patients with advanced stage ovarian cancer and observed that CD8+ and FoxP3+ T-cell intertumoral infiltration was an independent prognostic factor of disease-specific survival in ovarian cancer patients. They also observed a correlation between the Cd8+/FoxP3+ ratio and increased survival [16]. In this study, the Treg cell population was assessed through immunohistochemistry in paraffin embedded tissue using antiFoxP3 monoclonal antibodies. The presence of FoxP3-positive cells was positively associated with an advanced stage of the disease. However, poor differentiation and advanced stage of the tumor are well-known negative prognostic factors. The authors themselves noted that the FoxP3 expression is not exclusive to Treg cells and can also be present in activated effector T cells CD4+ and CD25- that do not have regulation activity [16]. Similarly, more FoxP3+cells were present in the poorly differentiated tumors. Moreover, the main result of this study was the positive influence of the intertumoral CD8+ T cell infiltration on survival. This correspondence linking increased cytotoxic activity of T cells and an increased survival accords with the studies of Curiel et al. and others [11]. For example, Zhang et al. [19] observed that the presence of intertumoral cytotoxic T cells correlated with improved clinical outcome and either delayed recurrence or delayed death in patients with advanced ovarian carcinoma [19]. The high level of CD3+ cytotoxic lymphocytes was proven to be linked with an increased expression of interferon-gamma, interleukin-2, and lymphocyte-attracting chemokines within the cancer nest [19]. Increased immune response linked with a depletion of Treg cells from the tumor microenvironment may be related to improved long-term outcomes in ovarian cancer patients. Treg cells infiltrate a cancer nest, and cancer development is related to an increased Treg cell level in the peripheral blood, ascites, and a microenvironment of cancer metastases such as the omentum [9, 20]. Increasing infiltration of Treg cells into a tumor site leads tothe suppression of an anti-cancer immune response in patients with various types of cancers [2]. Noordam et al. analyzed the overall Treg cell levels in the peripheral blood as these were shown to correlate with decreased survival in malignant pleural mesothelioma patients. However, when they divided the Treg population into naïve and effector cells, they observed that nTreg levels corresponded with improved survival rates [17]. Notably, Chung demonstrated that CD4+ T cell infiltration in patients with breast cancer is linked with improved survival rates, but breast cancer patients with a high percentage of FoxP3+/CD8+ within the CD4 + Tcells have decreased survival rates. Increasing the FoxP3+/CD4+ ration

of TIL seems to be an independent adverse prognostic factor in a hormone receptor positive group of patients, mainly in a luminal A subtype of breast cancer [21]. Analyses of patients with lung [7], urinary bladder [8] and ovarian cancers [9, 10] yielded similar results, confirming the relationship between Treg cell population size and cancer stage.

In our study, we analyzed the percentage of Treg cells (FoxP3+) in the overall T-cell population (CD4+ and CD25+) in the peripheral blood of ovarian cancer patients and found that an increased level of FoxP3+, CD4+, and CD25+ cells statistically significantly correlated with a worse prognosis.

Treg cells constitute a dynamic population of cells whose activity depends on multiple factors, including cytokines, chemokines, and others. Li et al. linked the poor prognosis with the fact that the infiltration of Treg cells to the cancer nest is associated with increasing transcript levels of IL-10 and TGFB1. This has been confirmed in ovarian cancer patients, among others [5]. High epithelial IL-6 expression is linked with worse survival rates in patients with ovarian cancer, and this cytokine is involved in the expansion of immune cells (mainly CD4+ cells andTreg cells [22]) in the tumor microenvironment [23, 24]. Furthermore, Toker et al. demonstrated that the Treg cells that infiltrate the ovarian cancer nest are typified by a distinct surface phenotype that differs from the ones found in other types of cancer nests. These cells are characterized by an increased expression of PD-1 and 4-1BB, both of which are associated with an increased suppressive capacity [25]. Additionally, Curiel et al. reported that, in the later stages of cancer, Treg cells seem to have a preference towards tumors and ascites and will only rarely accumulate in the draining lymph nodes [11].

Cancer surgery is necessary and life-saving; however, the majority of patients develop postoperative recurrence and metastases, which constitute the two main causes of cancer-related deaths. Rationally combining cancer surgery with immunotherapies to improve immune and treatment outcomes can transform an immunosuppressive effect into a therapeutic opportunity [26]. Various types of anticancer drugs have been designed to influence the Treg cell population in both thetumor microenvironment and the peripheral blood. For instance, it has been proven that Treg cell levels decline significantly in the peripheral blood of patients treated by radiofrequency ablation for advanced lung cancer [13]. Neoadjuvant chemotherapy (NACT) is also associated with the reduced infiltration of Treg cells in omental biopsy for advanced ovarian cancer in a group of patients responding to NACT. However, the enhanced immune response in the ovarian cancer microenvironment that should follow such changes in the Treg cell level is tempered by the increased level of PLD-1 and CTLA-4 expression [20].

Depending on the tumor microenvironment profile, the suppressor activity of Tregs can be redirected into a pro-inflammatory phenotype. The ovarian cancer nest is infiltrated by Treg cells [11] that have been found to be of a highly activated phenotype. Treg cells with strong suppressive activity can be found not only in cancer tissue, but also in the peripheral blood. Both populations are being extensively studied for their potential use in anticancer therapy. On the other hand, it is possible to instrumentally evoke and augment antitumor immunity in cancer patients by selectively depleting eTreg cells [27] (by blocking the chemokine CTLA-4 receptor) or by using the monoclonal antibody against the PD-1 receptor. Treg cells express the well-known immune checkpoint receptor PD-1, which reportedly marks "exhausted" Treg with lower suppressive function [28].

Treg cells are not only responsible for the evasion of cancer cells from immune surveillance, but also cooperate with other immune cells, including TAMs, which are predominantly from M2 subgroups [29, 30]. These subgroups are responsible for the secretion of TGF-beta and Il-10 that support the differentiation of Treg cells [31]. TAM may also interact with Treg cells in the tumor microenvironment, producing exosomes, such as miRNAs [4]. Targeting these exosomes might become a novel treatment for ovarian cancer [4]. Monoclonal antibodies against CCR4 are able to selectively reduce the Treg cell population. Chemokine axis CCL22-CCR4 plays a critical role in the recruitment of Tregs through CCR4 receptors that are present on Treg cells. Furthermore, anti-CCR4 mAb supports the efficacy of immunotherapy in ovarian cancer patients [27].

Cancer surgery is necessary and life-saving; however, the majority of patients develop postoperative recurrence and metastases, which constitute the main causes of cancer-related deaths [32]. Rationally combining cancer surgery with immunotherapies can transform the immunosuppressive effect into a therapeutic opportunity [26].

CONCLUSIONS

In sum, it is well-known that Treg cells may be a key component of the immunosuppressive tumor microenvironment that is responsible for tumor cell escape from immune surveillance [25]. The assessment of Treg levels prior to the start of chemotherapy is clinically useful and may predict overall survival in ovarian cancer patients.

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