FoxP3$^+$ Regulatory T Cells in Peripheral Blood of Patients with Epithelial Ovarian Cancer

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ABSTRACT

Background: Ovarian cancer is the fifth leading cause of death from malignancy in women. $CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cells are a subset of T lymphocytes with great inhibitory impact on immune response. **Objectives:** To investigate the percentage of $CD4^+CD25^+FoxP3^+$ regulatory T cells in the peripheral blood of the Iranian patients with epithelial ovarian cancer compared to healthy women and to evaluate the correlation of the Treg cell percentage with clinicopathological characteristics including cancer stage and CA-125 serum level. **Methods:** Seventeen women with epithelial ovarian cancer and 20 healthy subjects were enrolled in the study. Peripheral blood mononuclear cells were stained at the surface, for CD4 and CD25 molecules, followed by fixation, permeabilization and intracellular staining for FoxP3 molecule. After processing and flowcytometry analysis, prevalence of Treg cells was determined as the percentages of $CD25^+FoxP3^+$ cells among $CD4^+$ lymphocytes. **Results:** Despite no difference in the percentage of total $CD4^+$ lymphocytes, analysis indicated that Treg cell percentage was significantly higher in ovarian cancer patients than controls ($5.7 \pm 3.1\%$ versus $2.8 \pm 1.4\%$, $p=0.002$). A trend toward higher Treg cells was observed in higher stages of ovarian cancer (III+IV) in comparison to lower stages (I+II) ($6.5 \pm 3.2\%$ vs. $4.44 \pm 2.7\%$, $p=0.2$). Higher percentage of Treg cells was also observed in the patients with high CA125 (CA-125 >100 U/mL) in comparison to those with low CA-125 serum level (CA-125 $\leq$ 100 U/mL) although the difference was not significant (6.44 versus 4.18%, $p=0.19$). **Conclusion:** Increased frequency of Tregs in ovarian cancer might participate in immune suppression in these patients. The findings collectively suggest the likely impact of Treg cell–targeted immunotherapy in ovarian cancer.

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INTRODUCTION

Ovarian cancer is the fifth leading cause of cancer death in American women (1). This cancer is the 8th most prevalent cancer in Iran (2) and has been reported to have lower age-standardized incidence rate (ASR) in Iran compared to developed countries (3). As reported by WHO, the most common type of ovarian cancer is epithelial cancer; which accounts for more than 75% of ovarian cancers (4). Since its presenting symptoms are nonspecific, this cancer is usually diagnosed at advanced stages (III/IV) (5) and exhibits a high rate of cancer recurrence after first treatment regimen (6). New strategies seem, accordingly, to be required for both diagnosis and management of ovarian cancer.

Anti-tumor immune responses to tumor antigens are usually unable to restrict cancer progression as cancers evade immunological responses by organizing tolerance mechanisms in favor of its own progression. Regulatory T (Treg) cells are one of the main inhibitory arms of immunity, which reported to participate in cancer evasion in mouse models and human cancers (7). These cells contribute to immune tolerance through down-regulation of T lymphocyte activity and inhibition of antigen presenting cells (8). Both the number and the activity of $CD4^+CD25^+FoxP3^+$ Treg cells have been reported to be increased in different kinds of cancer (9-13). These observations raised the possibility that Treg cells participate in cancer pathogenesis. Curiel *et al*. reported that $CD4^+CD25^+FoxP3^+$ are recruited to the site of ovarian tumor under the influences of CCL22 chemokine, and are able to suppress antitumor immunity to ovarian cancer (14). In the current study, we investigated the percentage of CD4+CD25+FoxP3+ regulatory T cells in the peripheral blood of the Iranian patients with epithelial ovarian cancer compared to healthy women and evaluated the correlation of the Treg cell percentage with clinicopathological characteristics including cancer stage and CA-125 tumor marker status.

MATERIALS AND METHODS

Out of 40 women with pelvic mass that were highly suspicious for malignancy according to clinical and radiologic assessment, seventeen women with pelvic mass pathologically confirmed to have epithelial ovarian cancer were enrolled in this study. All the patients were new cases who received no prior treatment, chemotherapy and/or radiating therapy before sample collection. Samples with coagulate as well as those which stayed in ward or research lab more than 2 hours before staining were also excluded. Controls included 20 healthy women with normal pelvic sonography with no history of malignancy or autoimmune diseases among their first degree relatives. Controls were matched with patients group for age and parity. Informed consent was obtained from all subjects before sample collection. 6-8 mL EDTA-peripheral blood was obtained from the subjects before operation and diluted 1/1 with PBS before ficoll-hypaque density centrifugation. After centrifugation, the buffy coat containing peripheral blood mononuclear cells was harvested for further flowcytometry analysis.

For surface marker staining, 100 µl of cell solution containing 500000 cells was added to 5 µl of Anti-h-CD4 PerCP and Anti-h-CD25 FITC monoclonal antibody (BD Bioscience, USA) and incubated for 30 minutes at room temperature in dark. The appropriate isotype control antibodies (all from BD-USA) were simultaneously added to a separate tube containing the cells from the same patient and incubated for 30 minutes

in dark. After centrifugation and washing with the washing solution, the samples were fixed using 300 µl of 1% paraformaldehyde and permeabilized by the same amount of saponin 0.1% (Sigma-Germany). Anti-h-FoxP3 PE, was then added to permeabilized cells and the cells were incubated for more 30 minutes in dark. Appropriate isotype control was simultaneously used in the control tube with permeabilized cells. After final fixation, samples were analyzed immediately by using a BD FACSCalibur flowcytometer (BD-USA). Cellquest-pro software package (BD-USA) was used for data acquisition and analysis as previously described (12). Briefly the percentage of total $CD4^+$ T cells was firstly calculated in lymphocyte gate. In order to define the percentages of $FoxP3^+$ regulatory T cells among the lymphocyte population, the lymphocyte gate was applied on the quadrant of CD4/FSC dot plot. The $CD4^+$ positive lymphocyte gate was finally applied on the CD25/ $FoxP3^+$ dot plot and the percentage of $CD4^+CD25^+FoxP3^+$ lymphocytes was considered as regulatory T cells percentage (Figure 1). The information regarding CA-125 serum level was obtained from patients' files.

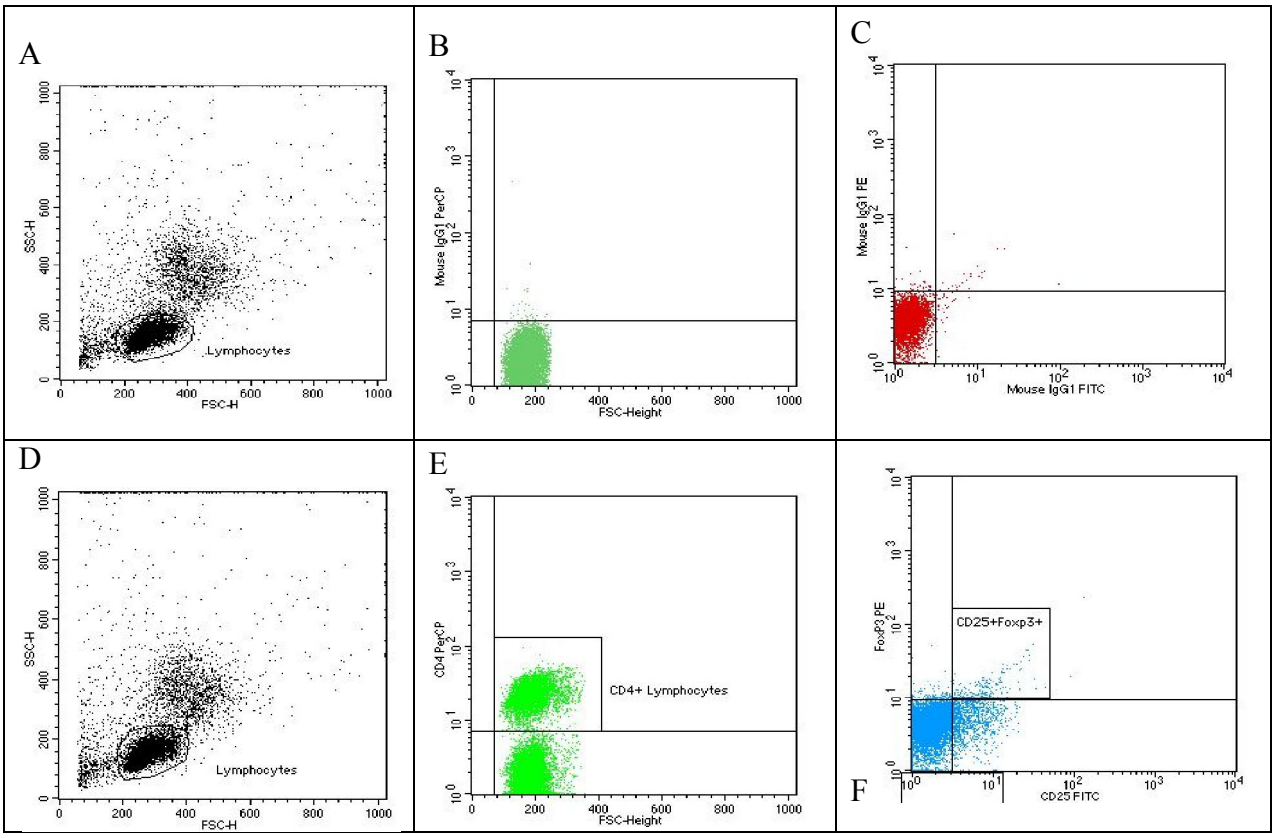

Figure 1. Flowcytometry analysis of $CD4^+CD25^+FoxP3^+$ cells in a sample stained for CD4, CD25 and FoxP3 markers and the cognate isotype controls. A to C are the dot plot of isotype control tube: lymphocytes were firstly gated on FSC/SSC dot plot (A), quadrant marker were then set on FSC/isotype control antibody-PerCP dot plot (B), as well as on isotype control antibody-FITC/ isotype control antibody-PE dot plot (C). D to F are the dot plots from the test tube: after gating the lymphocytes on FSC/SSC dot plot (D), quadrant from FSC/isotype ab-PerCP dot plat was applied and CD4+ cells were gated on FSC/CD4-PerCP plot (E). This gate was then applied on CD25-FITC/FoxP3-PE dot plot with a quadrant from isotype control antibody-FITC/isotype control antibody-PE dot plat to determine the percentage of CD4+CD25+FoxP3+ T cells.

Statistical analysis was performed by using SPSS software package (version 11.5; SPSS Inc, Chicago, IL, USA). Student T-test was used for comparing the data between groups. In all analysis P value less than 0.05 were considered statistically significant.

RESULTS

Seventeen patients with epithelial ovarian cancer as well as 20 healthy women as control group were matched for age and parity. Mean age of patient group was 50.3 ± 11.6 and the mean age of control group was 49.8 ± 8.0. Eight out of seventeen patients (47.1%) were diagnosed with pathological stage III. The reset of the patients were equally fall into stages I, II and IV, i. e. 3 out of 17 (17.6%) in each group.
The percentage of $CD4^+$ lymphocytes in patients was 40.6 ± 10.2 which was not significantly different from that in control subjects (42.7 ± 10.7, p=0.537, Figure 1). Despite of this , the percentage of CD4+CD25+ FoxP3+ T cells observed to be significantly increased in patients with epithelial ovarian cancer compare to healthy subjects (5.7 ± 3.1 vs. 2.8 ± 1.4, p=0.002, Figure 2).

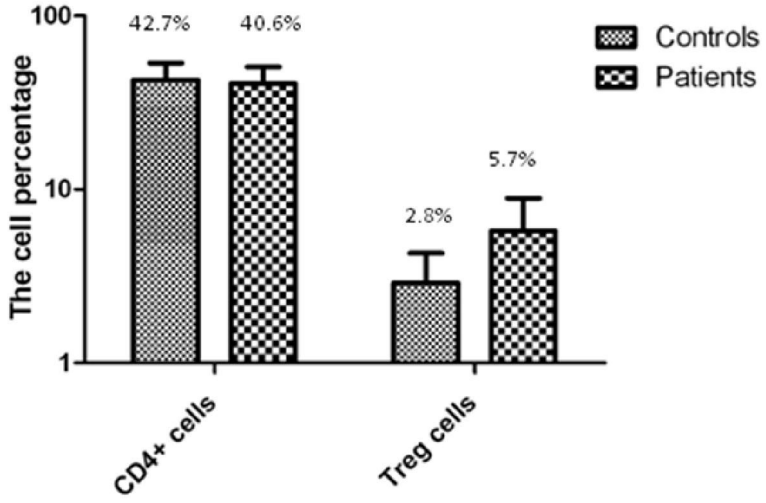

Figure 2. The percentage of $CD4^+$ T cells and $CD4^+CD25^+FoxP3^+$ Treg cells in patients with epithelial ovarian cancer and healthy control subjects.

Statistical analysis indicated a trend toward the increase of Treg cells percentage in higher stages (stages III+IV, N=11) in comparison with those in lower stages (Stages I+II, N=6); although the difference was not statistically significant (6.5 ± 3.2 vs 4.44 ± 2.7, respectively; p=0.2). After dividing the patients based on the concentration of CA-125 tumor maker (CA-125 ≤ 100 U/mL and CA-125 >100 U/mL), the trend toward higher percentage of Treg cells was observed in the patients with high CA-125 (CA-125 >100 U/mL) in comparison to those with low CA-125 (CA-125 ≤ 100 U/mL) although the difference was not significant (6.44% versus 4.18%, p=0.19)

DISCUSSION

Tumors have been indicated to employ their own mechanisms, as well as the host immune inhibitory system to escape immunity (15). $CD4^+CD25^+$ Treg cells have immunosuppressive characteristics (16) that are essential for maintaining self-tolerance and preventing autoimmunity in healthy condition. Antigen specific expansion of Treg cells has been indicated to induce transplantation tolerance (16) while depletion of Treg cells by monoclonal antibodies, in experimental animal models, has been reported to be associated with tumor eradication (17). Adoptive transfer of Treg cells diminished tumor-specific immunity and caused tumor progression (18). Accordingly, investigation of Treg cells in health and diseases, as well as their functional significant, have been the subjects of different studies in recent decade (19,20). In the older publications, the cells which co-expressed CD4 and high level of CD25 markers were considered as regulatory T cells (19,21). By the discovery of $FoxP3^+$ as the main specific marker of conventional $CD4^+$ $CD25^+$ regulatory T cells (22), recent studies have focused on this transcription factor and its functional significance in pathological conditions (20,22,23). In the present study, the percentages of total $CD4^+$ lymphocytes, as well as $CD4^+CD25^+FoxP3^+$ lymphocyte subset (Treg cells) were investigated in patients with epithelial ovarian cancer and healthy controls.

While the percentage of total $CD4^+$ lymphocytes was not different between patients and healthy control subjects, the prevalence of Treg subset among this T-lymphocyte subgroup; i.e. $CD4^+CD25^+$ $FoxP3^+$ cells, was observed to be significantly increased in patients with ovarian cancer compared to healthy subjects.

Anti-tumor immunity has been wildly reported to be compromised in ovarian cancer (24,25). After lymphocytes isolation from ascetic fluid of patients with primary and recurrent ovarian carcinoma, and culturing of the cells in the presence of IL-2, the numbers of $FoxP3^+$ regulatory T lymphocytes have been indicated to be increased but the percentage of $CD8^+$ T lymphocytes was decreased, especially in recurrent ovarian carcinoma (26). The finding suggests the presence of cellular immunity suppression in ovarian cancer in which regulatory T cells seems to be involved. In another study by Leveque *et al*, culture of Treg cells derived from epithelial ovarian cancer samples in the presence of IL-2 led to conversion of these inhibitory cells to pro-inflammatory Th17 lymphocytes; implying the probable role of cancer milieu in the accumulation of Treg cells in tumor microenvironment (27). These findings, along with our observation suggest that the increased regulatory T cell may provide immune inhibitory milieu in ovarian cancer patients and participate, at least partly, in immune compromisation reported in ovarian cancer.

Elevated Treg cells percentage is not restricted to ovarian cancer. High numbers of $CD4^+CD25^+$ Treg cells have been demonstrated in lung, pancreas, breast, liver, and skin cancer patients, either in the peripheral blood or around and within the tumor (28,32). Increase in the number and/or function of Treg cells has been already reported to be associated with cancer progression, as well as prognosis in the cancer patients. Recently, Niaragh and colleagues reported the increased frequency of regulatory T cells in progressive chronic lymphocytic leukemia (CLL) compared to indolent CLL patients (33). $FoxP3^+$ Treg cells might promote hepatocellular carcinoma (HCC) development through enhancing angiogenesis and decreasing CD8+ T cells. These cells are speculated to be a prognostic indicator for HCC (34). Infiltration of ICOS+ Treg cells in breast tumors correlates with a poor prognosis (35). Angiogenic status of renal cell

carcinoma (RCC) may be related with regulatory T cells presence in the tumor microenvironment (36). Recruitment of regulatory T cell to tumor and acetic fluid in patients with ovarian cancer has been indicated to be associated with reduced survival (14) and also with poor prognosis (37).

Previous investigations in cancer patients, have also demonstrated the stage-dependent increase of Treg cells and probable correlation with overall survival (38). In the present study, dividing the patients into low (I+II) and high (III+IV) stages showed a trend toward higher Treg cell percentage in high stages, although the difference between two groups was not significant ($p=0.2$). CA-125 is a tumor marker which is routinely used as a progression marker in ovary cancer (39). A non-significant higher percentage of Treg cells was also observed in the patients with high CA-125 (CA-125 >100 U/ml) in comparison to those with low CA125 (CA-125 $\leq$ 100 U/ml). The trend toward elevated Treg cells in higher stages and higher CA125 level collectively suggest that the rise in Treg cell percentage in ovarian cancer might be correlated with cancer progression, and most likely disease outcome and prognosis. This suggestion, however, needs to be confirmed in a large study with higher numbers of patients.

Immune system is regulated by a complex network consisting of various types of cells and molecules. As one of the main players of immune balance, immunotherapy targeted regulatory T cells attracted attention in recent decade. Antibodies to Treg cells such as ONTAK, was reported to deplete regulatory T cells from peripheral blood of patients with melanoma, and to enhance immune response (40). Findings of the present study suggest that the immunotherapy targeting these inhibitory cells may have promising results in ovarian cancer.

In conclusion, observing no change in the percentage of total $CD4^+$ lymphocytes but significant increase of Treg cell subset in ovarian cancer patients suggest the likely role of this cell subset in immune compromisation and, consequently, ovarian cancer progression. These findings may have implication in cell mediated-immunotherapy of ovarian cancer based on Treg cell inhibition. The limitation of the present study however should not be ignored. Increasing the sample size, and following up the patients may improve future studies.

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