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*Original Article*

# Increased frequency of regulatory T cells in the peripheral blood of patients with epithelial ovarian cancer

Li Li[1], Lihua Yang[2], Yeqing Lu[2], Yinghua Li[3], Fangfang Wang[1], Lu Chen[1]

*[1]Department of Gynecologic Oncology, Zhejiang Cancer Hospital, Hangzhou, Zhejiang Province, People's Republic of China; [2]Department of Gynecology, Yuhang Women's Hospital, Hangzhou, Zhejiang Province, People's Republic of China; [3]Women's Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang Province, People's Republic of China*

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**Abstract:** Ovarian carcinoma is one of the leading causes of cancer-related death among women. The purpose of this study was to investigate the frequency of $CD4^+CD25^+CD127^-$ regulatory T cells (Tregs) in the peripheral blood of patients with epithelial ovarian cancer (EOC) and to explore their immune regulatory roles. 118 female patients with EOC and 30 healthy women were recruited. The percentage of $CD4^+CD25^+CD127^-$ Tregs within the $CD4^+$ T cell population in the peripheral blood was detected by flow cytometry. The relation between clinicopathologic factors and Tregs frequency was analyzed. To study the immunoregulatory capacity of $CD4^+CD25^+CD127^-$ Tregs, cytokines produced by $CD4^+CD25^+CD127^-$ Tregs was measured by ELISA and the proliferation of cells was measured by incorporation of [$^3$H] thymidine. The percentage of $CD4^+CD25^+CD127^-$ Tregs within the $CD4^+$ T cell population in the peripheral blood of EOC patients was 5.34 ± 1.77%, significantly higher than that in healthy women. No correlation of Tregs frequency with tumor stage, differentiation grade and histological subtype was found. $CD4^+CD25^+CD127^-$ Tregs derived from peripheral blood did not produce IFN-γ, but large amounts of IL-10. The isolated $CD4^+CD25^+CD127^-$ Tregs notably decreased the proliferation of $CD4^+CD25^-$ T cells. We provide evidence of increased frequency of Tregs with potent immunosuppressive features in the peripheral blood of patients with EOC, which may be responsible for immune tolerance in EOC.

**Keywords:** Regulatory T cells, epithelial ovarian cancer, peripheral blood

## Introduction

Ovarian carcinoma is one of the leading causes of cancer-related death among women in China. The majority of women with ovarian cancer are diagnosed with advanced-stage disease and prognosis is generally rather poor [1]. As the emergence of a tumor results from the disruption of cell growth regulation and the failure of the host to provoke a sufficient immunological antitumor response, the immune system is increasingly becoming a target for intense research in order to study the host's immune response against ovarian cancer and has been reported in association with the development and progression of ovarian carcinoma.

Considerable evidences have confirmed that Regulatory T cells (Tregs), a functionally unique population of T cells characterized by coexpression of CD4 and CD25, maintain the peripheral immune tolerance. Recently, many studies have reported that Tregs are crucially involved in the immunoloregulation of cancer as they can suppress cancer immune response by direct contact and cytokine production [2]. Increased frequency of Tregs has been shown in the peripheral blood, tumor site and/or draining lymph nodes in a wide variety of human cancer, such as breast, prostate, lung, colon, liver and hematological malignancies, etc [3-6]. Many studies have had an agreement that increased frequency of Tregs in cancer patients is a prognostic implication for a worse outcome. On the other

**Table 1.** Clinicopathologic features of 118 patients with epithelial ovarian cancer

| | Number (%) |
|---|---|
| FIGO Stage | |
| I | 11 (9.32%) |
| II | 18 (15.25%) |
| III | 75 (63.56%) |
| IV | 14 (11.86%) |
| Differentiation grade | |
| G1 | 15 (12.71%) |
| G2 | 16 (13.56%) |
| G3 | 87 (73.73%) |
| Histological subtype | |
| Serous | 86 (72.88%) |
| Mucinous | 14 (11.86%) |
| Endometrioid | 13 (11.02%) |
| Clear cell | 5 (4.24%) |

hand, studies aimed at depleting or functional inhibition of Tregs in the experimental models have resulted in successful control of cancer. The deletion of Tregs in vivo enhances the tumor immunity in mouse model of cancer [7].

Several studies have reported the presence of Tregs in the tumor tissue, ascites and peripheral blood of patients with ovarian epithelial cancer [8-12]. The relationship between pathogenesis of Tregs and prognosis in ovarian cancer was first reported by Curiel and colleagues who showed that accumulation of intratumoral Tregs was associated with poor survival [8]. However, in subsequently published studies, the relationship between Tregs and clinicopathologic factors, such as tumor grade, stage, histology, suboptimal debulking and survival is still ambiguous and controversial. Wolf and colleagues showed that patients with low levels of intratumoral Foxp3 had substantially improved survival compared with patients with high levels [12]. But Barnett et al. reported high Tregs infiltration in ovarian cancer was associated with high grade, advanced stage and suboptimal debulking, but not with survival [9]. A latest study published in 2015 also showed there were no significant differences in the peripheral blood, peritoneal fluid, and tumor-infiltrating Tregs percentage based on tumor stage grade, or histology [11]. Although no agreement on the clinical significance of Tregs in ovary cancer, such as association with prognosis and as markers of disease progress, all the findings have agreed that regulatory T cells are definitely involved in the tumor immunity suppression in ovarian cancer [13].

In the present study, we tried to explore the immune regulatory roles of Tregs in the peripheral blood of patients with epithelial ovarian cancer (EOC). We used flow cytometry to determine the relative proportion of Tregs cells in the peripheral blood of patients with EOC and healthy women. Furthermore, we performed functional analysis to confirm their suppressive function.

## Material and methods

### *Subjects*

A total of 118 ovarian cancer patients admitted to the Department of Gynecologic Oncology, between August 2012 and June 2015. 30 Female healthy volunteers were also recruited as control group. The study was approved by the Ethical Committee of Zhejiang Cancer Hospital. Written informed consent was obtained from all subjects. No subject had received radiotherapy, chemotherapy, or other medical interventions previously and none had any other concurrent primary malignancy. 20 ml of heparinised peripheral venous blood was obtained before surgery. Then ovarian cancer patients were submitted to staging operation. After the staging operation, the surgical specimens were examined carefully by two experienced pathologists. Each case of ovarian cancer was evaluated for clinical and pathological parameters, including surgical stage, histological subtype and differentiation grade. The differentiation grades of ovarian cancer included grade I, grade II, and grade III. Staging was performed according to FIGO (International Federation of Gynecology & Obstetrics) classification. All subjects had not received hormone or immunosuppressant therapy for at least past 6 months.

### *Flow cytometry*

In brief, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient separation. Then, prepared PBMCs were incubated with FITC-conjugated anti-CD4, PECy7-conjugated anti-CD25, PE-conjugated anti-CD127 (BD Biosciences; USA) or isotype controls according to the manufacturer's instructions. To determine the percentage of Tregs, the $CD4^+$ population was gated first, followed by the $CD25^+CD127^-$ populations. FoxP3,

**Table 2.** The percentage of $CD4^+CD25^+CD127^-$ Tregs within the $CD4^+$ cell population in the peripheral blood

| *Group* | $CD4^+CD25^+CD127^-$ Tregs/ $CD4^+$ T cells (%) |
|---|---|
| Epithelial Ovarian Cancer (n=118) | 5.34±1.77* |
| Healthy controls (n=30) | 3.34±0.84 |

*Compared with control, P<0.05.

a member of the forkhead or winged helix family of transcription factors, is considered as the most reliable marker for Tregs. For validation experiments, intracellular FoxP3 staining was measured and performed on cells stained with anti-CD4, anti-CD25, and anti-CD127. Cells were fixed and permeabilized using a fix/perm kit (eBioscience; USA) according to the manufacturer's instructions, then labeled with APC-conjugated anti-FoxP3 antibody or its isotype control antibody. $1\times10^5$ cells were acquired and data were analyzed on an FC500 flow cytometer (Beckman Coulter) with CXP software. High levels of FoxP3 were detected in $CD4^+CD25^+CD127^-$ cells (>90%).

*Cell proliferation assay*

Freshly isolated $CD4^+CD25^+CD127^-$ Tregs ($1\times10^5$ cells) were incubated with autologous $CD4^+CD25^-$ T cells ($1\times10^5$ cells) on anti-CD3 mAb (10 ng/ml, DAKO)-coated 96-well round-bottomed plates in the presence of an anti-CD28 mAb (10 μg/ml; PharMingen, CA) for 72 hours. The proliferation of cells was measured by incorporation of $[^3H]$ thymidine. After 16 hours, cells were harvested and thymidine incorporation was expressed as cpm.

*Cytokine production assay*

Freshly isolated $CD4^+CD25^+CD127^-$ Tregs or $CD4^+CD25^-$ T cells ($1\times10^5$ cells) were placed on anti-CD3 mAb (10 ng/ml, DAKO)-coated 96-well flat-bottomed plates and cultured in 200 μl of AIM-V medium (Life Technologies, Inc.) at 37°C for 24 hours. Then the supernatants were harvested and tested for cytokine production with Quantikine human IFN-γ or IL-10 ELISA kit (R&D Systems, MN) according to the manufacturer's instructions.

*Statistical analysis*

Analysis was performed using SPSS version 16.0. All data were expressed as mean ± SD. Comparison between two groups was done using Student's *t*-test for normally distributed variables. When more than two groups of data sets were compared, one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test was performed. The association between clinical prognostic parameters and Tregs expression was assessed using Spearman's rho test. P<0.05 was considered significant.

## Results

*Clinical and pathological characteristics of the subjects*

Clinicopathologic features of 118 patients with epithelial ovarian cancer were shown in **Table 1**. Results were available from 118 patients and 30 controls. The mean age of patients with ovarian cancers and healthy controls was 54.60 ± 9.22 years, 51.58 ± 6.83 years, respectively. No significant difference of age was found between two groups. Each case of ovarian cancer was evaluated for clinical and pathological parameters, including surgical stage, histological subtype and differentiation grade.

*Increased populations of $CD4^+CD25^+CD127^-$ Tregs in the peripheral blood of patients with ovarian cancer*

As shown in **Table 2**, the percentage of $CD4^+CD25^+CD127^-$ Tregs within the $CD4^+$ T cell population in the peripheral blood of ovarian cancer patients was 5.34 ± 1.77%, significantly higher than that in healthy women (3.34 ± 0.84, P< 0.05, **Table 2**). Representative flow cytometry pictures for Tregs percentage in both groups were shown in **Figure 1**. There was no significant difference of $CD4^+CD25^+CD127^-$ Tregs frequency among ovarian cancer patients according to tumor stage, differentiation grade or histological subtype (P>0.05). No correlation of Tregs frequency with tumor stage (r=0.31, P= 0.14), differentiation grade (r=0.27, P=0.35) and histological subtype (r=0.09, P=0.78) was found.

*$CD4^+CD25^+CD127^-$ Tregs suppressed the proliferation of $CD4^+CD25^-$ T cells*

To study the immunoregulatory capacity of $CD4^+CD25^+CD127^-$ Tregs from ovarian cancer patients, coculture experiments were performed. $CD4^+CD25^+CD127^-$ Tregs notably decreased the proliferation of $CD4^+CD25^-$ T cells. But there was no difference in the suppressive activity of

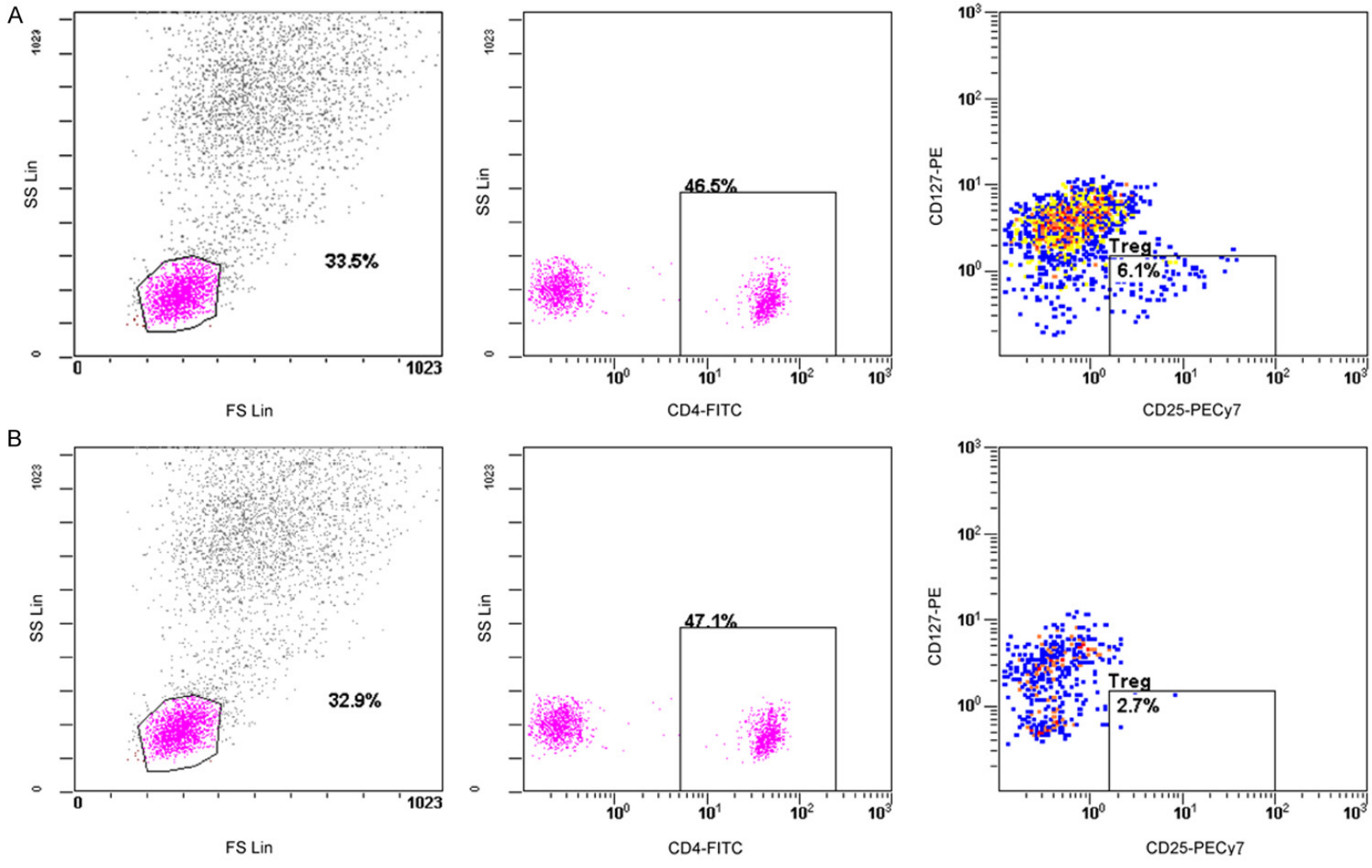


**Figure 1.** Flow cytometry. The percentage of $CD4^+CD25^+CD127^-$ Tregs within the $CD4^+$ T cell population in the peripheral blood was detected by flow cytometry. Prepared PBMCs were incubated with FITC-conjugated anti-CD4, PECy7-conjugated anti-CD25, PE-conjugated anti-CD127 or isotype controls according to the manufacturer's instructions. The $CD4^+$ population was gated first, followed by the $CD25^+CD127^-$ populations. A. The percentage of $CD4^+CD25^+CD127^-$ Tregs within the $CD4^+$ T cell population in the peripheral blood of one EOC patient was 6.1%. B. The percentage of $CD4^+CD25^+CD127^-$ Tregs within the $CD4^+$ T cell population in the peripheral blood of one healthy woman was 2.7%.

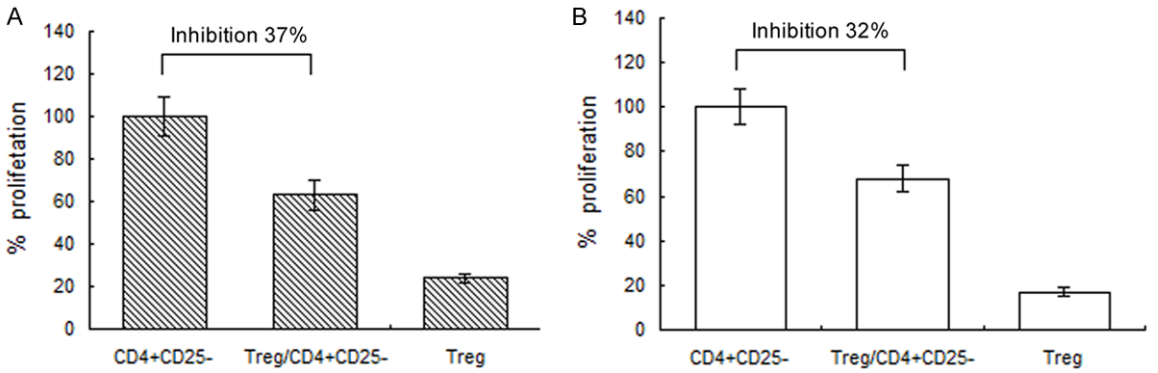


**Figure 2.** Cell proliferation assay. Purified $CD4^+CD25^+CD127^-$ Tregs isolated from the peripheral blood of EOC patients and healthy controls were incubated with autologous $CD4^+CD25^-$ T cells (ratio of 1:1) and the proliferation of cells was measured in triplicate cultures by [$^3$H] thymidine incorporation. The proliferation of $CD4^+CD25^-$ T cells alone, or $CD4^+CD25^+CD127^-$ Tregs alone was also measured. The proliferation of $CD4^+CD25^-$ T cells was considered as 100%. The isolated $CD4^+CD25^+CD127^-$ Tregs notably decreased the proliferation of $CD4^+CD25^-$ T cells. But there was no difference in the suppressive activity of Tregs between EOC patients (A) and healthy controls (B).

Tregs between ovarian cancer patients (**Figure 2A**) and healthy controls (**Figure 2B**).

*$CD4^+CD25^+CD127^-$ Tregs produced large amounts of IL-10 but no IFN-γ*

$CD4^+CD25^+CD127^-$ Tregs and $CD4^+CD25^-$ T cells were stimulated with immobilized anti-CD3 mAbs respectively and the supernatants were examined for IFN-γ and IL-10 level. As shown in **Figure 3A**, $CD4^+CD25^+CD127^-$ Tregs isolated from ovarian cancer patients did not produce IFN-γ, whereas $CD4^+CD25^-$ T cells secreted IFN-γ. Moreover, $CD4^+CD25^+CD127^-$ Tregs isolated from ovarian cancer patients produced large amounts of IL-10, but $CD4^+CD25^-$ T cells secreted a little IL-10. Similarly, $CD4^+CD25^+CD127^-$ Tregs isolated from healthy women produced large amounts of IL-10 but did not produce IFN-γ (**Figure 3B**). There was no difference in the level of IL-10 production of Tregs between ovarian cancer patients and healthy controls.

## Discussion

We investigated the frequency of Tregs in the peripheral blood of patients with EOC and performed functional analysis to confirm their suppressive function in the present study. To our knowledge, this study contained the largest samples reporting the circulating Tregs in EOC patients although not the first. We found significantly increased frequency of Tregs in the peripheral blood of EOC patients. However, in contrast to other researcher's findings, there was no correlation of Tregs frequency with tumor stage, differentiation grade or histological subtype. Furthermore, we confirmed that $CD4^+CD25^+CD127^-$ Tregs had a suppressive function by evaluating cytokine production and suppressive capacity against $CD4^+CD25^-$ T cells.

In this study, we used flow cytometry to investigate circulating Tregs and found that circulating $CD^{4+}CD25^+CD127^-$ cells were more frequent in women with EOC compared to healthy women. For validation experiments, intracellular FoxP3 staining was measured and showed high levels of FoxP3 in $CD4^+CD25^+CD127^-$ cells. Most Tregs are defined based on coexpression of CD4, CD25, and the transcription factor, FoxP3. FoxP3 is considered as a major marker and functional regulator of Tregs development and function [14]. However, as intracellular FoxP3 staining may injury cells, $CD4^+CD25^+CD127^-$ phenotype has been proposed as an alternative to identify Tregs in clinical samples [15]. Here, we demonstrated that $CD4^+CD25^+CD127^-$ cells could be recognized as a highly purified population of $FoxP3^+$ Tregs in EOC patients and healthy women.

However, when we explored the relation between clinicopathologic factors and Tregs frequency in the peripheral blood of patients with EOC, no correlation of Tregs frequency with tumor stage, differentiation grade and histological subtype was found, in accordance with Wertel and colleagues' finding, but opposite

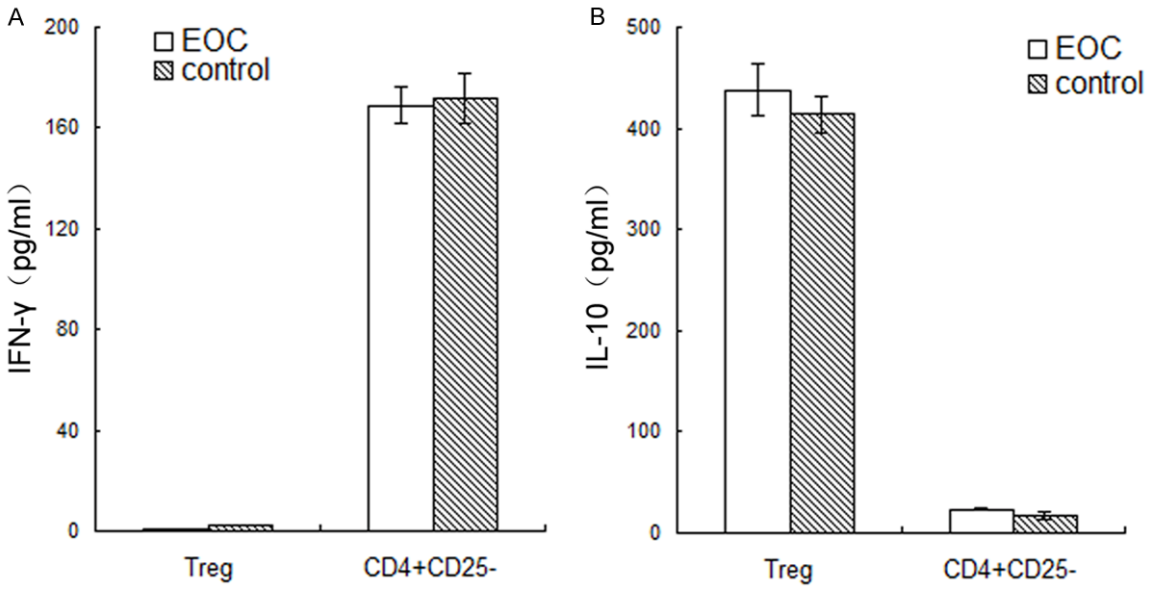


**Figure 3.** Cytokines production assay. $CD4^+CD25^+CD127^-$ Tregs and $CD4^+CD25^-$ T cells isolated from the peripheral blood of EOC patients and healthy controls were stimulated with immobilized anti-CD3 mAbs respectively and their supernatants were examined for IFN-γ and IL-10 level by ELISA. A. $CD4^+CD25^+CD127^-$ Tregs derived from EOC patients or healthy women did not produce IFN-γ. Both $CD4^+CD25^-$ T cells secreted IFN-γ. There was no difference in the level of IFN-γ production of $CD4^+CD25^-$ T cells between EOC patients and healthy controls. B. $CD4^+CD25^+CD127^-$ Tregs derived from EOC patients produced large amounts of IL-10. Similarly, $CD4^+CD25^+CD127^-$ Tregs derived from healthy women produced large amounts of IL-10. There was no difference in the level of IL-10 production of Tregs between EOC patients and healthy controls. Both $CD4^+CD25^-$ T cells secreted a little IL-10. There was no difference in the level of IL-10 production of $CD4^+CD25^-$ T cells between EOC patients and healthy controls.

with other researchers' findings [8, 9, 11, 12]. No agreement among researchers on the relation between the Tregs frequency in the peripheral blood and clinicopathologic factors might be attributed to age of subjects and different sample size. Our study contained 118 EOC women and 30 healthy women. No significant difference of age was found between two groups. The relation between age and human circulating Tregs has been investigated in several studies and Tregs function is thought to be age-dependent [16]. However, there have been no agreement on whether Tregs frequency in the peripheral blood increases with age or not [17, 18]. To ascertain the true relation between EOC and Tregs frequency, it is important that future work aims to recruit more samples and an appropriately matched control population with respect to clinicopathologic factors. On the other hand, as other researchers reported significantly higher percentage of Tregs in the ascites than peripheral blood of ovarian cancer patients and presumed that peripheral blood Tregs are specifically recruited into PF and tumor tissue [8, 11], perhaps it is more important to monitor Tregs in local microenvironment rather than circulating Tregs in EOC. But the reason for the accumulation of Tregs in the ascites and tumor tissue has not been clearly explained yet.

Anti-tumor immunity of Tregs has been wildly reported to be compromised in ovarian cancer [19, 20]. Tregs have immunosuppressive characteristics and depletion of Tregs by monoclonal antibodies has been reported to be associated with tumor eradication [21, 22]. Adoptive transfer of Tregs caused tumor progression while deletion of Tregs in vivo enhances the tumor immunity in mouse model of cancer [7, 23]. The number of Tregs increased but the percentage of $CD8^+$ T cells decreased after lymphocytes isolated from ascites of patients with primary and recurrent ovarian carcinoma and cultured of the cells in the presence of IL-2 [13]. The finding suggests the presence of cellular immunity suppression in ovarian cancer in which Tregs seems to be involved. Leveque et al. reported culture of Tregs derived from EOC samples in the presence of IL-2 resulted in conversion of these inhibitory cells to pro-inflammatory Th17 lymphocytes, implying the possi-

ble role of cancer milieu in the accumulation of Tregs in tumor microenvironment [24]. In the present study, we determine the functional capacity of Tregs isolated from EOC patients and healthy women. $CD4^+CD25^+CD127^-$ Tregs derived from EOC patients or healthy women produce no IFN-γ, but large amounts of IL-10. There was no difference of the level of IL-10 production between EOC patients and healthy women. IFN-γ can promote Th1 cell differentiation and possess direct cytotoxic and cytostatic activity toward cancer cells by promoting cytotoxic activity of T cells and NK cells [25]. Like other inflammatory cytokines, active IFN-γ inhibits the peripheral generation of $FoxP3^+$ Tregs from naive $CD4^+$ T cells [26]. We showed Tregs secreted no IFN-γ whereas $CD4^+CD25^-$ T cells secreted IFN-γ. IL-10 is a cytokine produced by Tregs and can both directly and indirectly inhibit effector T cells responses during cancer, autoimmunity and infection [27-29]. Though also expressed by other immune cells, deletion of IL-10 in Tregs resulted in the development of spontaneous colitis, and exaggerated immune responses at other environmental interfaces such as the lung and skin [30]. Tumor-derived $CD4^+CD25^+$ Tregs can suppress DCs function by producing IL-10 [31]. In the present study, large amounts of IL-10 production confirmed suppressive function of Tregs. Then, we found $CD4^+CD25^+CD127^-$ Tregs isolated from EOC women suppressed the proliferation of $CD4^+$ $CD25^-$ T cells. Our findings suggest that Tregs play an essential role in the antitumor immune of patients with EOC. Host immune surveillance decreases during tumorigenesis, association with increased proportion of circulating Tregs, even if many tumor-infiltrating lymphocytes are observed in the tumor mass. Not only $CD4^+CD25^+CD127^-$ Tregs can suppress the proliferation and activation of $CD8^+$ cytotoxic cells, $CD4^+$ T cells, NK cells, and NKT cells, but also inhabit the antigen presentation function of DCs [2, 32, 33]. Therefore, depletion Tregs, or inhibition function of Tregs, may be an ideal target for improving anticancer immune responses.

In conclusion, the results of our study showed increased frequency of Tregs in the peripheral blood of patients with EOC and confirmed their suppressive function. We presume that Tregs may play a role in controlling the anti-tumor immune against EOC. But no correlation of Tregs frequency with tumor stage, differentiation grade and histological subtype was found in the present study. Perhaps it is more valuable to monitor Tregs in local microenvironment rather than circulating Tregs in EOC. Deep insight into the specific role of Tregs in EOC is the key for targeting them in a way that is beneficial for the clinical outcome.

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## Disclosure of conflict of interest

None.

**Address correspondence to:** Lu Chen, Department of Gynecologic Oncology, Zhejiang Cancer Hospital, Hangzhou 310022, Zhejiang Province, People's Republic of China. E-mail: zq1990876@sina.com

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