Increased frequency of regulatory T cells in the peripheral blood of patients with endometrioid adenocarcinoma

LI LI[1,2], YINGHUA LI[3], ZHUOMIN YIN[1], JING ZHU[1], DINGDING YAN[1] and HANMEI LOU[1,2]

[1]Department of Gynecologic Oncology, Zhejiang Cancer Hospital;
[2]Key Laboratory of Radiation Oncology of Zhejiang Province, Hangzhou, Zhejiang 310022;
[3]Women's Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310006, P.R. China

Abstract. The aim of the present study was to investigate the frequency of cluster of differentiation (CD)4+CD25+CD127- regulatory T cells (Tregs) in the peripheral blood mononuclear cells (PBMCs) of patients with endometrioid adenocarcinoma (EA). A total of 82 female patients with EA were recruited. The PBMCs were flow cytometrically analyzed to determine the percentage of CD4+CD25+CD127- Tregs within the CD4+ T cell population. The associations between the prevalence of Tregs in PBMCs and defined clinical prognostic parameters were evaluated. To study the immunoregulatory capacity of Tregs, the level of specific cytokines were detected by ELISA, and the proliferation of cells was analyzed by incorporation of ^{3}H-thymidine. It was revealed that Treg/CD4+ ratio in the peripheral blood of patients with EA was 4.89±1.42%, significantly higher than Treg/CD4+ ratio in healthy women. No correlation was observed between Treg frequency and stage, grade of differentiation, menopausal status or age. CD4+CD25+CD127- Tregs secreted large amounts of IL-10 but not IFN-γ. The level of IL-10 secreted by Tregs from patients with EA and healthy controls was not significantly different. In addition, there was no significant difference in the suppressive activity of Tregs in patients with EA compared with that of the healthy controls. These findings demonstrate that the increased frequency of immunosuppressive Tregs in patients with EA may be responsible for immune tolerance in endometrial cancer.

Correspondence to: Professor Hanmei Lou, Department of Gynecologic Oncology, Zhejiang Cancer Hospital, 1 East Banshan Road, Gongshu, Hangzhou, Zhejiang 310022, P.R. China
E-mail: zjhzlouhanmei@sina.com

Abbreviations: Tregs, regulatory T cells; EA, endometrioid adenocarcinoma

Key words: Treg cells, endometrial cancer, endometrioid adenocarcinoma, peripheral blood, interleukin-10

Introduction

In China, endometrial cancer is one of the most common malignant carcinomas of the female reproductive organs, and its incidence has steadily increased (1). The vast majority of endometrial cancer cases are endometrioid adenocarcinoma (EA) cases (2,3). This type of uterine cancer forms in the glandular cells of the uterine lining (2). EA is commonly detected early and has a high cure rate (4,5). A number of risk factors are involved in the etiology of endometrial cancer, including obesity, diabetes and ageing of the population (6). It is believed that tumorigenesis results from cell growth dysregulation and the failure of the host to provide a sufficient antitumor immune response (7). The immune system, which is markedly influenced by age and certain hormones, has been reported to be associated with the development and progression of endometrial cancer (6). Immune cells, including tumor-associated macrophages, cluster of differentiation (CD)8+ cytotoxic T cells, natural killer (NK) cells, CD4+ T helper cells and dendritic cells (DCs) constitute an important part of the microenvironment and are thought to be critical for endometrial cancer development and progression (6). However, the precise mechanisms by which the immune system is modulated in patients with endometrial cancer remain poorly understood.

Regulatory T cells (Tregs), characterized by the co-expression of CD4 and CD25, have been revealed to suppress T cell-mediated host immune responses against self- and non-self-antigens (8-10). The transcription factor Forkhead box protein P3 (FOXP3) is considered to be one of the most specific markers of Tregs. Evidence has confirmed that Tregs are involved in the immune tolerance of malignant neoplasms, as they suppress immune responses by cytokine secretion and direct contact (11). Increased levels of Tregs have been reported at tumor sites, draining lymph nodes and in the peripheral blood in many kinds of human cancer, including breast, colon, lung, prostate, liver, ovarian cancer and hematological malignancies (12-16). An increased level of Tregs in human cancer is considered to be a poor prognostic factor. Studies have attempted to reduce the number of Tregs in order to control cancer in experimental models; Turk *et al* (17) demonstrated enhanced tumor immunity in mouse models by depletion of Tregs.

Some researchers have reported the presence of Tregs in the tumor tissue, peripheral blood and tumor draining lymph nodes of patients with endometrial cancer (18-21). High levels of intratumoral were revealed to associate with poorer disease-free survival, increased grade of differentiation, cancer stage, the extent of lymph node metastases and myometrial invasion (18,20). The mechanisms underlying Treg enrichment in patients with endometrial cancer are not clear; the proportion and the role of Tregs in the peripheral blood of patients with endometrial cancer is still ambiguous and controversial, as a limited number of small studies have been reported (18,21). The increased number of Tregs in the peripheral blood of patients might be responsible for suppressing anti-tumor immunity.

In the present study, to elucidate the role of Tregs in EA, the frequencies and suppressive functions of Tregs in the peripheral blood of patients with EA and healthy controls were evaluated. The association between the frequency of Tregs in the peripheral blood of patients with EA and certain clinical prognostic parameters was also analyzed.

Materials and methods

Study participants. The present study was approved by the Ethical Committee of Zhejiang Cancer Hospital (Hangzhou, China). Written informed consent was obtained from all participants. The current study included a total of 82 female patients with EA who were admitted to the Department of Gynecologic Oncology at Zhejiang Cancer Hospital between August 2012 and June 2015, in addition to 30 healthy women who were recruited during routine health checkups at Zhejiang Cancer Hospital, enrolled as the controls. The age range of the patients with EA was 30-70 years, while the age range of the healthy controls was 31-69 years. All patients with EA were diagnosed by endometrial biopsy prior to recruitment. At the recruitment stage, none of the patients had received chemotherapy, radiotherapy or any other previous medical intervention. Prior to surgery, 20 ml heparinized peripheral venous blood was obtained, and the patients with EA underwent subsequent staging surgery; the surgical specimens were examined by experienced pathologists. Clinical and pathological parameters, including age, menopausal status, stage, grade of differentiation, lymphatic or vascular permeation and lymph node metastatic status were evaluated for each patient. The surgical staging of EA was performed according to the International Federation of Gynecology and Obstetrics (FIGO) classification (22). None of the participants had received hormonal or immunosuppressant therapy for ≥6 months prior to staging. A total of 20 ml heparinized peripheral venous blood was also obtained from healthy controls.

Flow cytometry. A total of 20 ml peripheral venous blood was collected from each participant into sterile heparinized container. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (GE Healthcare Life Sciences, Uppsala, Sweden) density gradient separation for 30 min at 800 x g at 20°C. The PBMCs were washed twice in PBS and then resuspended in PBS at $1x10^6$ cells/ml for further analysis. PBMCs were analyzed by flow cytometry for the phenotypic characterization of Tregs. In brief, PBMCs were

Table I. Percentage of $CD4^+CD25^+CD127^-$ Tregs within the $CD4^+$ cell population in the peripheral blood.

Group	$CD4^+CD25^+CD127^-$ Tregs/$CD4^+$ T cells (%)
Endometrioid adenocarcinoma (n=82)	4.89±1.42[a]
Healthy controls (n=30)	3.34±0.84

[a]P=0.019 vs. healthy controls. CD, cluster of differentiation; Treg, regulatory T cell.

incubated with specific antibodies (1:10 dilution; mouse anti-human, monoclonal; Beckman Coulter, Brea, CA, USA) against phycoerythrin-cyanine 5-conjugated anti-CD4 (cat. no. A07752), fluorescein isothiocyanate-conjugated anti-CD25 (cat. no. IM0478U) and phycoerythrin-conjugated anti-CD127 (cat. no. IM1980 U), or isotype controls (1:10 dilution; IgG1-FITC, cat. no. A07795; and IgG1-PE, cat. no. A07796; Beckman Coulter), in the dark for 30 min at 20°C. Subsequently, a washing step was performed using PBS. For blocking, cells were suspended in 0.2% (w/v) BSA (cat. no. 554657; BD Biosciences, San Jose, CA, USA) with Human BD Fc Block™ (cat. no. 564220; BD Biosciences) for 10 min at 20°C. Data from $1x10^5$ cells/sample were acquired using the FC500 flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed with Win 7 CXP version 2.2 software (Beckman Coulter). To determine the relative ratio of Tregs, the $CD4^+$ population was gated, followed by the $CD25^+CD127^-$ population. FOXP3 is widely accepted as the most reliable marker for Tregs, thus validation experiments were performed by intracellular FOXP3 staining with the Anti-Human Foxp3 Staining Set APC kit (cat. no. 77-5776-40; eBioscience; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol; $CD4^+CD25^+CD127^-$ cells with high expression of FOXP3 (>90%) were confirmed.

Cell culture, cytokine and proliferation assays. PBMCs from the peripheral venous blood of patients with EA and healthy controls were isolated by Ficoll-Hypaque density gradient separation. $CD4^+CD25^+CD127^-$ Tregs and $CD4^+CD25^-$ T cells were purified with the MACS CD4 Multisort kit (cat. no. 130-055-101), CD25 (cat. no. 130-092-983) and CD127 (cat. no. 130-094-945) Microbeads (Miltenyi Biotec, Auburn, CA, USA) using magnetic separation columns, according to the manufacturer's protocol. The purities of the enriched cells were >92% (determined by flow cytometry). Purified $CD4^+CD25^+CD127^-$ Tregs and $CD4^+CD25^-$ T cells ($1x10^5$ cells) were placed on anti-CD3 mAb (10 ng/ml; cat. no. M725429-2; DAKO; Agilent Technologies, Inc., Santa Clara, CA, USA)-coated 96-well flat-bottomed plates and cultured in 200 μl AIM-V medium (Thermo Fisher Scientific, Inc.) at 37°C for 24 h. $CD4^+CD25^+CD127^-$ Tregs and $CD4^+CD25^-$ T cells were stimulated with immobilized anti-CD3 mAbs. The supernatants were harvested and the level of IFN-γ and IL-10 production was detected using the Human IFN-γ Quantikine ELISA kit (cat. no. DIF50; R&D Systems, Inc., Minneapolis MN, USA) and the Human IL-10 Quantikine
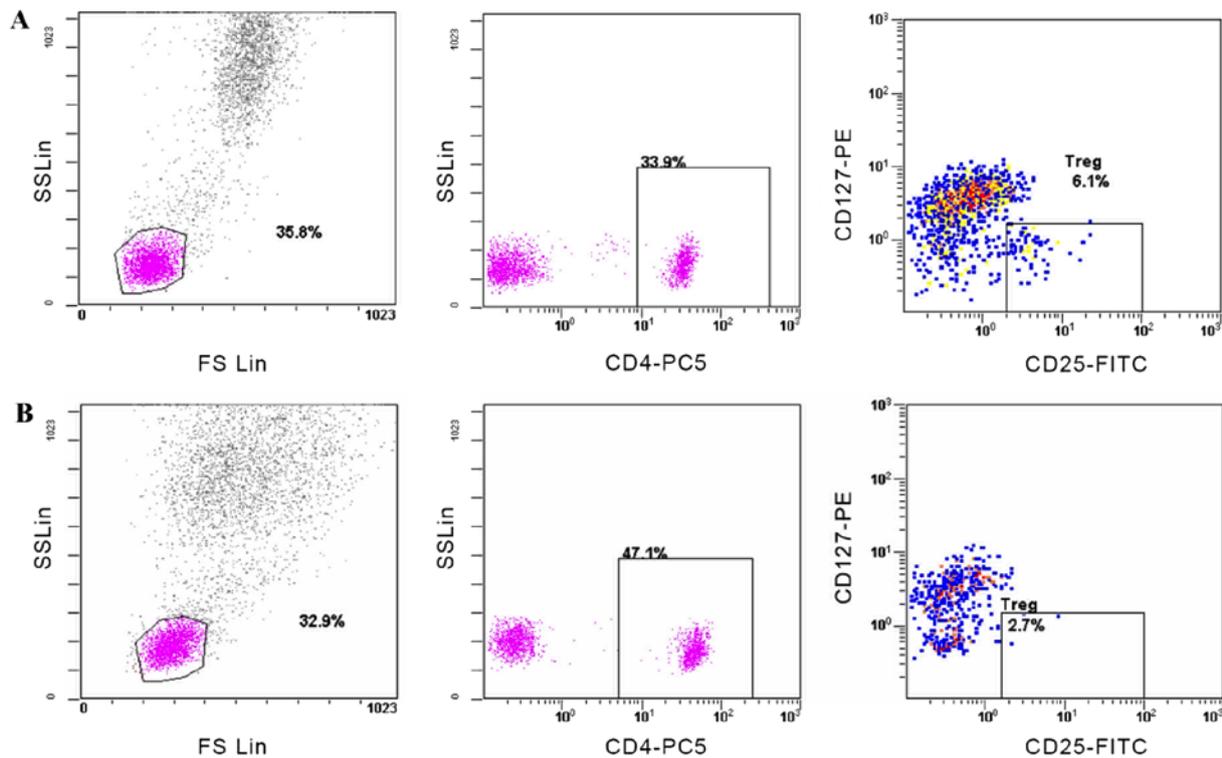
Figure 1. Flow cytometry. The percentage of CD4+CD25+CD127- Tregs within the CD4+ T cell population in the peripheral blood was detected by flow cytometry. (A) The percentage of CD4+CD25+CD127- Tregs within the CD4+ T cell population in the peripheral blood of one patient with endometrioid adenocarcinoma 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%. CD, cluster of differentiation; Treg, regulatory T cell; PC5, phycoerythrin-cyanine 5; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

ELISA kit (cat. no. D1000B; R&D Systems, Inc.), according to the manufacturer's protocols. Subsequently, the anti-proliferative function of CD4$^+$CD25$^+$CD127$^-$ Tregs was assessed by evaluating the proliferative activity of CD4$^+$CD25$^-$ T cells in response to anti-CD3 plus anti-CD28 antibodies, in the presence of autologous CD4$^+$CD25$^+$CD127$^-$ cells. Briefly, purified CD4$^+$CD25$^+$CD127$^-$ Tregs were incubated with autologous CD4$^+$CD25$^-$ T cells (1x10^5 cells each) on anti-CD3 mAb (10 ng/ml)-coated 96-well round-bottomed plates in the presence of an anti-CD28 mAb (10 μg/ml; cat. no. 555726; BD Biosciences) for 72 h. Proliferation was measured by incorporation of ^{3}H-thymidine (10 uCi/ml). Cells were harvested after 16 h and thymidine incorporation was measured using a scintillation counter and expressed as counts per minutes.

Statistical analysis. All data were expressed as the mean ± standard deviation. All experiments were performed at least three times. Comparisons between two groups were analyzed using the Student's t-test for normally distributed variables. ANOVA followed by Bonferroni correction was performed for more than two groups of data. Spearman's rank correlation coefficient test was used to analyze the association between clinical prognostic parameters and Treg frequency. P<0.05 was considered to indicate a statistically significant difference. Analyses were performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA).

Results

Clinical characteristics. All endometrial cancer cases were classified as EA. The mean age of the patients with EA was 53.65±7.06 years, whilst the mean age of healthy controls was 51.58±6.83 years; though this difference was not significant. A total of 28 patients with EA were <50 years old, while the other 54 patients were ≥50 years old. Among the 82 patients, 22 were premenopausal and 60 were postmenopausal.

Increased Treg/CD4$^+$ ratio in the peripheral blood of patients with EA. The CD4$^+$CD25$^+$CD127$^-$ Treg/CD4$^+$ ratio in the peripheral blood of patients with EA was 4.89±1.42%, significantly higher compared with that in healthy controls (3.34±0.84; P=0.019; Table I). Representative flow cytometry plots illustrate the percentage of Tregs in both groups (Fig. 1). As presented in Table II, no significant difference in CD4$^+$CD25$^+$CD127$^-$ Treg frequency was observed among patients with EA in association with tumor stage (P=0.753) or differentiation grade (P=0.686). As endometrial cancer occurs predominantly in postmenopausal women (23), 82 patients with EA were divided into postmenopausal (n=60) and premenopausal (n=22) groups; no significant difference in CD4$^+$CD25$^+$CD127$^-$ Treg frequency was observed in association with menopausal status (P=0.581). As the majority of women reach the menopause at ~50 years of age (1), CD4$^+$CD25$^+$CD127$^-$ Treg frequency between EA patients <50 years-old and those ≥50 years-old was compared; no significant difference was observed (P=0.667). No association between Treg frequency and tumor stage, grade of differentiation, menopausal status or age was observed.

Cytokines produced by CD4$^+$CD25$^+$CD127$^-$ Tregs. As illustrated in Fig. 2, CD4$^+$CD25$^+$CD127$^-$ Tregs derived from

Table II. Association between Treg frequency and clinico-pathological factors.

Clinicopathological factor	CD4$^+$CD25$^+$CD127$^-$ Tregs/CD4$^+$T cells (%)	P-value
Stag		0.753
I (n=59)	4.90±1.45	
II (n=8)	4.88±1.47	
III (n=15)	4.88±1.45	
Grade		0.686
G1 (n=17)	4.89±1.46	
G2 (n=42)	4.89±1.45	
G3 (n=23)	4.91±1.41	
Menopausal status		0.581
Premenopausal (n=22)	4.87±1.43	
Postmenopausal (n=60)	4.90±1.46	
Age, years		0.667
<50 (n=28)	4.86±1.43	
≥50 (n=54)	4.89±1.43	

CD, cluster of differentiation; Treg, regulatory T cell.

patients with EA produced little IFN-γ, but large amounts of IL-10. CD4$^+$CD25$^-$ T cells derived from both groups secreted large amounts of IFN-γ but notably less IL-10. Similarly, CD4$^+$CD25$^+$CD127$^-$ Tregs derived from healthy controls secreted little IFN-γ, but large amounts of IL-10. There was no significant difference in IL-10 secretion by Tregs between the two groups (P=0.274).

Treg suppression assays. As presented in Fig. 3, it was confirmed that CD4$^+$CD25$^+$CD127$^-$ Tregs markedly suppress the proliferation of CD4$^+$CD25$^-$ T cells. However, no significant difference was observed in the suppressive activity of Tregs between patients with EA (suppressive efficacy, 38%; Fig. 3A) and healthy controls (suppressive efficacy, 32%; Fig. 3B; P=0.196).

Discussion

The present study indicated that patients with EA have increased Treg frequencies in their peripheral blood compared with healthy controls. In line with a previous study (15), a significantly increased CD4$^+$CD25$^+$CD127$^-$ Treg/CD4$^+$ ratio in the peripheral blood of patients with EA with a suppressive function was revealed. These CD4$^+$CD25$^+$CD127$^-$ Tregs produced high levels of IL-10 and suppressed the proliferation of CD4$^+$CD25$^-$ T cells. However, there was no correlation with Treg frequency in association with stage, histological grading, menopausal status and age, which was in contrast to other studies (18,21).

FOXP3 is considered as the most specific marker for Tregs and is co-expressed with CD4 and CD25 (24). However, another study has reported that intracellular FOXP3 staining may cause cell damage (25), thus the CD4$^+$CD25$^+$CD127$^-$ phenotype

has been proposed as an alternative to identify Tregs in clinical samples. In the present study, it was confirmed that CD4$^+$CD25$^+$CD127$^-$ cells may be recognized as a highly purified population of FOXP3$^+$ Tregs.

However, in the present study, no correlation between Treg frequency and stage, histological grade, menopausal status or age was observed. Notably, some contrasting results were revealed when compared with other similar studies. It is widely accepted that tumor stage, grade of differentiation and myometrial invasion are independent prognostic factors for endometrial cancer. Chang *et al* (18) reported that the high prevalence of CD4$^+$CD25$^+$ Tregs in the cancer stroma and in the peripheral blood of 57 patients with endometrial cancer was closely associated with clinical features, including tumor grade, stage, lymph node metastasis and myometrium invasion. Sawan *et al* (26) revealed an increased proportion of Tregs in the peripheral blood of 24 patients with endometrial cancer, which may have been attributed to their postmenopausal status or age, but not with cancer stage or grade. The lack of agreement among researchers on the associations between the Treg frequency in the peripheral blood and clinical prognostic parameters may be attributed to different cancer types and/or different sample sizes. In the present study, all of the 82 samples collected were EA, whilst patients with non-endometrioid adenocarcinoma (NEA) were excluded. Different mechanisms are involved in the pathogenesis of EA and NEA (3,27). It was therefore hypothesized that the inhibition of the immune response in patients with endometrial cancers may be influenced by several inhibitory mechanisms, including the number of Tregs in the peripheral blood, the tumor microenvironment and the cancer type.

The association between age and circulating Tregs in humans has been investigated in numerous studies (26,28-30), and the function of Tregs is thought to be age-dependent (25). However, there is conflicting evidence as to whether Treg frequency in the peripheral blood increases with age or not (29,30). A number of studies concerning Tregs in the peripheral blood of patients with cancer did not investigate the association between age and Treg frequency, and more studies did not report the age of the control group (11,12,15,31). Although others provided this data, the healthy controls were significantly younger compared with patients (26,28-30). A lack of age-matched controls in such studies may mean that their results may be misleading. The present study investigated the effect of aging on Treg frequency by analyzing the correlation between age and Treg frequency; the mean age of patients with EA was similar to the healthy controls, and the difference was not significant. As endometrial cancer usually occurs in postmenopausal women (23), and women in China predominantly reach the menopause at ~50 years of age (1), CD4$^+$CD25$^+$CD127$^-$ Treg frequency between EA patients <50 years old and those ≥50 years old was compared; however, no statistically significant difference was observed. Therefore, differences in Treg frequency between patients with EA and healthy women were not associated with age.

To the best of our knowledge, there has been only one study (21) regarding the role of menopause in Treg frequency or function in patients with EA. Menopause is characterized by cessation of ovarian functions, including hormonal
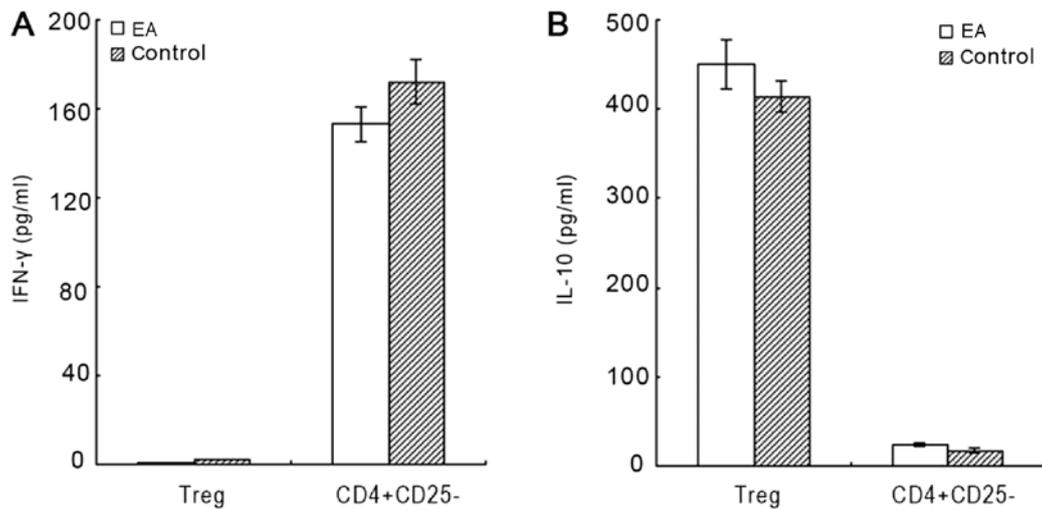
Figure 2. Cytokine production assay. CD4$^+$CD25$^+$CD127$^-$ Tregs and CD4$^+$CD25$^-$ T cells isolated from the peripheral blood of patients with EA and healthy controls were stimulated with immobilized anti-CD3 monoclonal antibodies respectively, and their supernatants were assessed by ELISA for IFN-γ and IL-10. (A) CD4$^+$CD25$^+$CD127$^-$ Tregs derived from patients with EA or healthy women did not produce IFN-γ, whilst the CD4$^+$CD25$^-$ T cells of both groups secreted IFN-γ. There was no difference in the level of IFN-γ production in CD4$^+$CD25$^-$ T cells between patients with EA and healthy controls. (B) CD4$^+$CD25$^+$CD127$^-$ Tregs derived from patients with EA 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 patients with EA and healthy controls. CD4$^+$CD25$^-$ T cells from both groups secreted little IL-10. There was no difference in the level of IL-10 production of CD4$^+$CD25$^-$ T cells between patients with EA and healthy controls. EA, endometrioid adenocarcinoma; CD, cluster of differentiation; Treg, regulatory T cell; IFN, interferon, IL-10, interleukin-10.
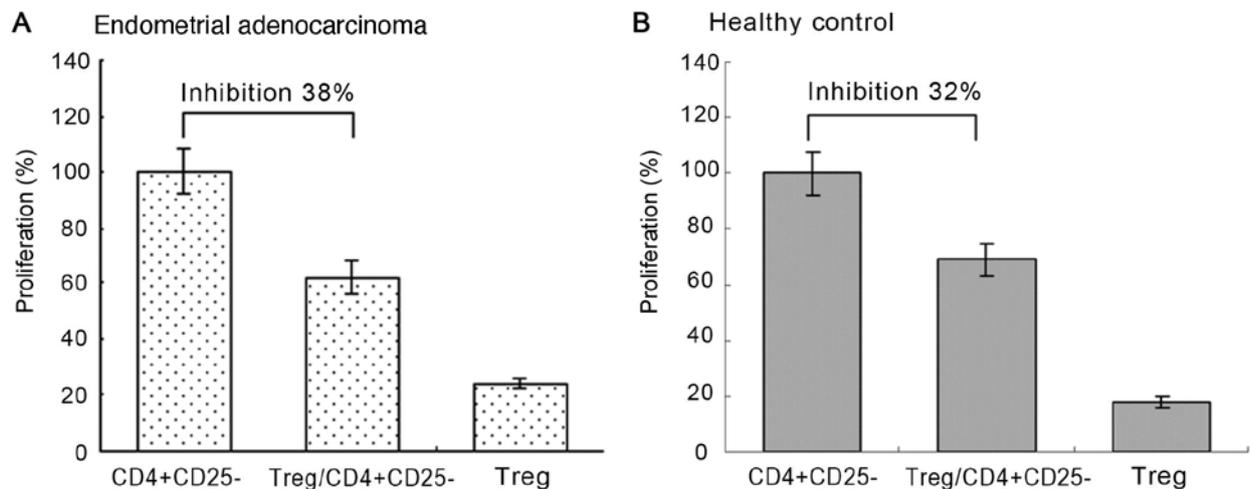

Figure 3. Cell proliferation assay. Purified CD4$^+$CD25$^+$CD127$^-$ Tregs isolated from the peripheral blood of patients with EA and healthy controls were incubated with autologous CD4$^+$CD25$^-$ T cells (ratio of 1:1) and cell proliferation was measured in triplicate cultures by ^{3}H-thymidine incorporation. 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 to be 100%. The isolated CD4$^+$CD25$^+$CD127$^-$ Tregs notably decreased the proliferation of CD4$^+$CD25$^-$ T cells; though there was no difference in the suppressive activity of Tregs between (A) patients with EA and (B) healthy controls. EA, endometrioid adenocarcinoma; CD, cluster of differentiation; Treg, regulatory T cell.

release, representing the end of a woman's reproductive life. The majority of cases of endometrial cancer are diagnosed in post-menopausal patients when blood estrogen levels are low, and >80% of cases are EA (6,27). Increased exposure to estrogen is considered to be an important risk factor of EA, and previous studies (32,33) have reported that Treg frequency changed during the menstrual cycle; the number of Tregs increased in the proliferative phase when compared with that in the secretory phase (32,33). In addition, estrogen has been reported to induce Treg proliferation *in vitro* (1). In the present study, a higher proportion of patients with EA were postmenopausal. However, there was no association between

Treg frequency in the peripheral blood and menopausal status in either patients with EA or in healthy women. Therefore, the observed difference in Treg frequency between patients with EA and healthy women did not appear to associate with menopausal status. These findings were in contradiction to a previous study (21). In order to investigate the true association between EA and Treg frequency, a larger cohort of patients is required with appropriately matched controls.

IFN-γ is an inflammatory cytokine that can exhibit direct cytotoxic and cytostatic activity toward tumor cells, and inhibits the peripheral induction of naive CD4$^+$ T cells to FOXP3$^+$ Tregs (34,35). IL-10, a cytokine produced by Tregs,

can directly and indirectly inhibit effector T cell responses in cancer (36-38). It is suggested that IL-10 production by Tregs may mediate immune suppression in the human tumor microenvironment. Tumor-derived Tregs are reported to suppress DC function by producing IL-10 (39). The results of the present study suggest that Tregs produced large amounts of IL-10, confirming their suppressive activity. Tumorigenesis is correlated with increased prevalence of Tregs in the peripheral blood (8). Previous studies have reported that Tregs suppress the antigen presentation function of DCs, the proliferation and activation of CD8$^+$ T cells, CD4$^+$ T cells, NK cells and NKT cells (11,40,41). In patients with melanoma, Tregs can regulate tumor-specific CD4$^+$ T cell responses. Therefore, it was hypothesized that increased Treg frequency in patients with EA may be responsible for impaired cellular immunity. It was revealed that CD4$^+$CD25$^+$CD127$^-$ Tregs suppress the proliferation of CD4$^+$CD25$^-$ T cells, which subsequently confirmed that CD4$^+$CD25$^+$CD127$^-$ Tregs isolated from patients with EA suppressed the T cell response. However, the level of IL-10 secreted by Tregs from patients and healthy controls was not significantly different. There was also no significant difference in the suppressive activity of Tregs between patients and healthy controls. It was therefore concluded that the suppressive function of Tregs might be relatively stable in different situations, though the number of Tregs alters in patients with EA, which may influence the regulation of the antitumor immune response by direct and indirect contact with other immune cells.

In conclusion, an increased frequency of Tregs with suppressive function in patients with EA may serve a role in regulating the antitumor immune response. However, no correlation between Treg frequency and tumor stage, differentiation grade, menopausal status or age was observed. As peripheral Tregs may be recruited to tumor sites and proliferate as a result of self-antigen recognition by memory Tregs, it may be more informative to monitor tumor-infiltrating Tregs in patients with EA, rather than circulating Tregs.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LL and HL contributed to the conception of this study and performed the preliminary experiments. All authors participated in the design of the study and implemented the research. DY and LL enrolled participants in the study and collected clinical data. JZ performed the flow cytometry experiments. LL, YL, ZY and JZ determined cytokine levels and cell proliferation. All authors participated in the statistical analysis and contributed to the interpretation of the results as well as the writing of the study. All authors reviewed all data and approved the final manuscript.

Ethics approval and consent to participate

This study followed international and national regulations in accordance with the Declaration of Helsinki. The present study was approved by the Ethical Committee of Zhejiang Cancer Hospital (Hangzhou, China). Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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