Interaction between Treg cells and tumor-associated macrophages in the tumor microenvironment of epithelial ovarian cancer

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Abstract. Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy. Inflammatory cells in the EOC microenvironment play a key role in tumor progression. In the present study, we investigated the mechanism of the accumulation of regulatory T cells (Tregs) induced by interleukin-10 (IL-10) derived from tumor-associated macrophages (TAMs) in the EOC microenvironment. The frequency of Tregs and TAMs was detected by immunofluorescence in 40 EOC tissues and 20 benign ovarian tumors, as well as the expression of IL-10 which was assessed by immunohistochemistry. It was found that the frequency of Treg cells and TAMs was significantly higher in the EOC than those in the benign ovarian tumors. The expression of IL-10 was also found to be higher in the EOC than that in the benign tumors. EOC patients with a high frequency of Tregs exhibited a significantly shorter overall survival time compared to those with a low frequency of Tregs. In addition, the expression of IL-10 in ascites and blood serum and the IL-10 released in the co-cultured system supernatants were detected by ELISA. Following $CD4^+$ T-cell co-culturing with macrophages and IL-10, it was observed by flow cytometric analysis that the frequency of Treg cells was increased in the presence of IL-10. It was also established that IL-10 released in the co-cultured supernatants was increased. We also detected the mechanism of Treg cells induced by IL-10 *in vivo*. The SKOV3 cell tumor volume and weight were much higher in the presence of IL-10 in a mouse subcutaneous model. These data suggest that IL-10 secreted by TAMs increase the frequency of Treg cells through the activation of Foxp3 during T-cell differentiation and promotes tumor progression.

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Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy (1). Over 70% of EOC patients are diagnosed with distant metastasis and their 5-year survival rate is less than 30% (2). Surgery and platinum-based chemotherapy are the main clinical treatments for most advanced EOC patients. However, most patients eventually experience cancer recurrence. Thus, an immunosuppressive EOC microenvironment should be overcome and new therapeutic approaches, such as immunotherapy, are needed.

Recently, many studies have shown that chronic inflammation plays a significant role in tumor progression (3). Many macrophages are found to infiltrate the tumor tissue in many solid malignancies. These tumor-associated macrophages (TAMs) are known to be recruited from peripheral blood monocytes by chemokines (4) and are involved in tumor progression (5). Many studies indicate that TAMs are associated with poor prognosis in various types of cancers (6-8). TAMs predominantly exhibit an M2 phenotype, which plays an important role in tumor angiogenesis, aggressiveness and modifies the tumor microenvironment. TAMs have the ability to produce high amounts of cytokines, which may contribute to tumor formation and invasion (9).

The interleukin (IL) family is a group of inflammatory cytokines. The major secretory characteristic of TAMs includes the production of interleukin-10 (IL-10), which is an immunosuppressive cytokine which participates in the growth and metastasis of different human malignant tumors. Sources of IL-10 include monocytes, a subtype of dendritic cells which activates macrophages and TAMs (10). IL-10 plays an important role in preventing inflammation in the negative feedback loop (11). The expression of IL-10 has been evaluated in many solid tumors, such as esophageal squamous cell, non-small cell lung and hepatocellular carcinoma (12-14).

Regulatory T cells (Tregs), a subpopulation of $CD4^+$ T lymphocytes, may suppress antitumor functions and regulate immune tolerance (15,16). Some studies have demonstrated that Treg cells can differentiate from naïve $CD4^+$ T cells in the presence of soluble factors such as TGF-β and IL-10 (17). However, the significance of IL-10 secreted by TAMs and its mechanism in regulating T cells in EOC remains unknown. In the present study, we aimed to explore the relationship between IL-10 and the accumulation of Treg cells in EOC and

investigated the mechanism by which TAMs induce Treg cell expression through IL-10.

Materials and methods

Patients. A total of 40 EOC samples and 20 benign ovarian tumor samples, obtained from the Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine (Shanghai, China) between January 2013 and 2015, were included in the present study (Table I). Peripheral blood mononuclear cells (PBMCs) were provided by the Shanghai Blood Center (Shanghai, China). Ethical approval for the present study was obtained from the Institutional Review Board of the Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine.

Immunofluorescence. Specimens were fixed in 4% neutral buffered formalin, embedded in OCT and cut into 8-μm sections. Treg cells were analyzed by immunofluorescence using double staining of CD4 and Foxp3. TAMs were analyzed using CD68. BSA (5%) (Sigma-Aldrich, St. Louis, MO, USA) was used to block non-specific binding. Then, sections were incubated with primary antibodies (anti-CD4, anti-Foxp3 and anti-CD68; Abcam, Cambridge, MA, USA) in a humidified chamber overnight at 4˚C. After being washed, the secondary anti-mouse IgG (for CD4; Invitrogen Life Technologies, Carlsbad, CA, USA) and anti-rabbit IgG (for Foxp3 and CD68; Invitrogen Life Technologies) were added for 45 min at room temperature. All reagents were used except the primary antibodies as negative controls. Sections were analyzed with a fluorescence microscope (Leica, Germany). Treg cells and TAMs were microscopically quantified by counting five fields of view at a magnification of x400.

ELISA assays for the detection of inflammatory factors in ascites and blood serum. Ascites samples of 16 EOC patients and blood serum samples of 7 healthy subjects were collected and stored at -80˚C. Subsequently, they were used for quantification of IL-10, IL-2 and IL-12 expression levels by ELISA assays according to the manufacturer's instructions (RayBiotech, Inc., Atlanta, GA, USA).

Immunohistochemistry. IL-10 was analyzed by immunohistochemistry. Sections were incubated in 3% hydrogen peroxide to block endogenous peroxide activity. BSA (5%) was used to block non-specific binding. After being washed, the sections were incubated with primary antibodies (mouse anti-human IL-10; Abcam) overnight at 4˚C in a humidified chamber. A secondary antibody (goat anti-mouse IgG) was added to the sections and DAB kits (both from Vector Laboratories, Inc., Burlingame, CA, USA) were used. All reagents were used except for the IL-10 as negative controls. The IL-10 content was microscopically observed at a magnification of x200 and x400.

Detection of IL-10 in TAMs and T-cell co-cultured supernatants. $CD4^+$ T cells and fresh peripheral blood $CD14^+$ monocytes were purified with immunomagnetic beads (Miltenyi Biotec Inc., Cambridge, MA, USA) from PBMCs provided by the Shanghai Blood Center. Cells (10^7) were cultured with 20 μl anti-CD4 beads (anti-CD14 beads) for 20 min at 4˚C. Then, the cells were resuspended in 800 μl buffer and the suspension was applied onto the column. The M1 macrophages were induced by M-CSF (10 ng/ml; R&D Systems, Inc., Minneapolis, MN, USA), LPS (500 ng/ml; Sigma-Aldrich) and IFN-r (20 ng/ml; R&D Systems). The M2 macrophages were induced by M-CSF (10 ng/ml) and IL-4 (20 ng/ml; both from R&D Systems). $CD4^+$ T cells were co-cultured with M1/M2 macrophages for 3 days and then the IL-10 concentration in the supernatant was detected using an IL-10 ELISA kit (RayBiotech). After 30 min, the optical density was detected at 450 nm using a microplate reader.

Table I. Clinical characteristics of the 60 studied patients.

Variables	n=60	Percent (%)
Diagnostic categories		
Benign ovarian tumors	20	33.33
Epithelial ovarian cancer	40	66.67
Histologic type		
Serous	24	60.00
Non-serous	16	40.00
Tumor grade		
Grade I	1	2.50
Grade II	18	45.00
Grade III	21	52.50
Clinical stage		
Stage I	11	27.50
Stage II	9	22.50
Stage III	19	47.50
Stage IV	1	2.50

Quantitative real-time PCR. The RNA was extracted from T cells co-cultured with the M1 group and M2 group using TRIzol. Takara Retroviral Reverse Transcriptase kit (Takara Bio, Inc., Otsu, Japan) was used to synthesize cDNA following the manufacturer's instructions. Primers for the Foxp3 gene were designed as follows: forward, 5'-CCCTCAACTGAGAA CTCAAGTC-3' and reverse, 5'-AGGTTGGCACCATAGTCT CCA-3'.

Western blotting. After 3 days of co-culture, $CD4^+$ T cells from the control, M1 and M2 group were treated with total protein lysis buffer (DingGuo Biotechnology Co., Ltd., Shanghai, China). Detection of Foxp3 was performed with a primary antibody against Foxp3 (1:200; Abcam) by western blotting.

The co-cultured system building in flow cytometric analysis of Treg cells. The macrophages were induced only by M-CSF (10 ng/ml; R&D Systems). Moreover, $CD4^+$ T cells were incubated with Mϕ macrophages/Mϕ macrophages + IL-10 (200 pg/ml) in a 6-well plate (10^6 cells/well) for 3 days. T cells were pre-stimulated with anti-CD28 (5 μg/ml) and anti-CD3 (10 μg/ml; both from BD Biosciences, USA) for 3 days. After being co-cultured with macrophages and IL-10,

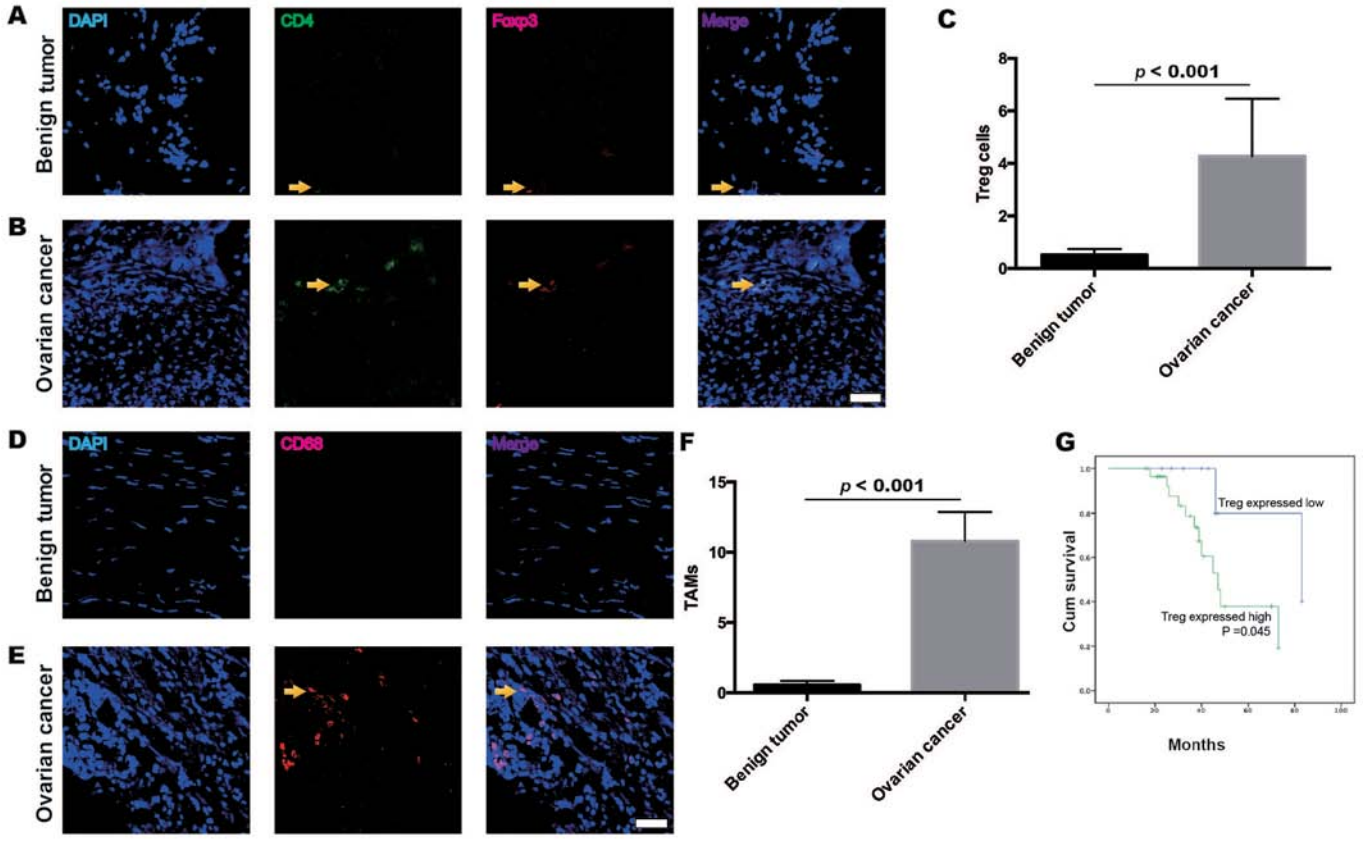

Figure 1. The frequency of Treg cells and TAMs in EOC samples and in benign ovarian tumor samples. (A) Tregs were stained with DAPI (blue), CD4 (green) and Foxp3 (pink) in the benign ovarian tumor tissues. (B) Tregs were stained with DAPI, CD4 and Foxp3 in the EOC samples; Scale bar, 50 μm. (C) The frequency of Tregs was significantly higher in the EOC samples than that in the benign ovarian tumor tissues. (D) TAMs were stained with DAPI (blue) and CD68 (pink) in the benign ovarian tumor tissues. (E) TAMs were stained with DAPI and CD68 in the EOC samples; Scale bar, 50 μm. (F) The frequency of TAMs was significantly higher in the EOC samples than that in the benign ovarian tumor tissues. (G) Kaplan-Meier curves (log-rank test) displaying overall survival of patients with low and high frequencies of Treg cells. Tregs, regulatory T cells; TAMs, tumor-associated macrophages; EOC, epithelial ovarian cancer.

the proportion of $CD4^+Foxp3^+$ Treg cells was detected by flow cytometric analysis. Firstly, the cells were stimulated with a T-cell stimulation cocktail and stained extracellularly with CD4 Pe-Cy5.5 (eBioscience, Inc., San Diego, CA, USA) for 45 min at 4˚C. Then, the cells were fixed and infiltrated with Perm/Fix solution (eBioscience) for 30 min. Subsequently, the cells were intracellularly stained with anti-Foxp3-allophycocyanin (APC) (eBioscience) for 45 min at 4˚C. All aforementioned steps were protected from light. Data were analyzed by a fluorescence-activated cell sorter (FACS).

The SKOV3 mouse subcutaneous model. The human EOC cells SKOV3-luciferase (SKOV3-Luc) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics. Peripheral blood $CD4^+$ T cells were purified from PBMCs with immunomagnetic beads. Female nude mice (5 weeks of age, 15 g) were purchased from Tongji University School of Medicine, Shanghai, China and bred under SPF conditions; three groups were mixed with the SKOV3-Luc cells for the establishment of the mouse model (four mice per group). Nude mice from each group were injected with 0.1 ml of RPMI-1640 medium containing $1x10^6$ SKOV3-Luc cells and $1x10^5$ $CD4^+$ T cells with or without IL-10. Tumor weight, tumor volume and total tumor flux were recorded. The SKOV3-Luc tumors *in vivo* were tested for Luc expression by D-luciferin (100 mg/kg; Life Technologies, Grand Island, NY, USA) and captured with the IVIS Spectrum (PerkinElmer, Inc., Waltham, MA, USA) for observation. The animal experiments were approved by the Medical Animal Care Committee and all the animals were cared for and only used in the laboratory.

Data and statistical analysis. Continuous data were evaluated using the LSD test, Levene's test and the Mann-Whitney U test. Overall survival was evaluated using Kaplan-Meier curves (log-rank test). Data are presented as the mean ± standard deviation. Figures are presented as the mean ± standard error of the mean. The statistical analysis was carried out using SPSS 22.0 and GraphPad Prism 6 software. P<0.05 was considered statistically significant.

Results

The frequencies of Treg cells and TAMs in the EOC samples are higher than those in the benign ovarian tumor samples. A total of 20 tissue samples from benign ovarian tumors and 40 tissue samples from EOC were analyzed with immunofluorescence confocal microscopy to evaluate the frequency of Treg cells (Fig. 1A-C) and TAMs (Fig. 1D-F). Significant differences were identified between the frequency of Treg

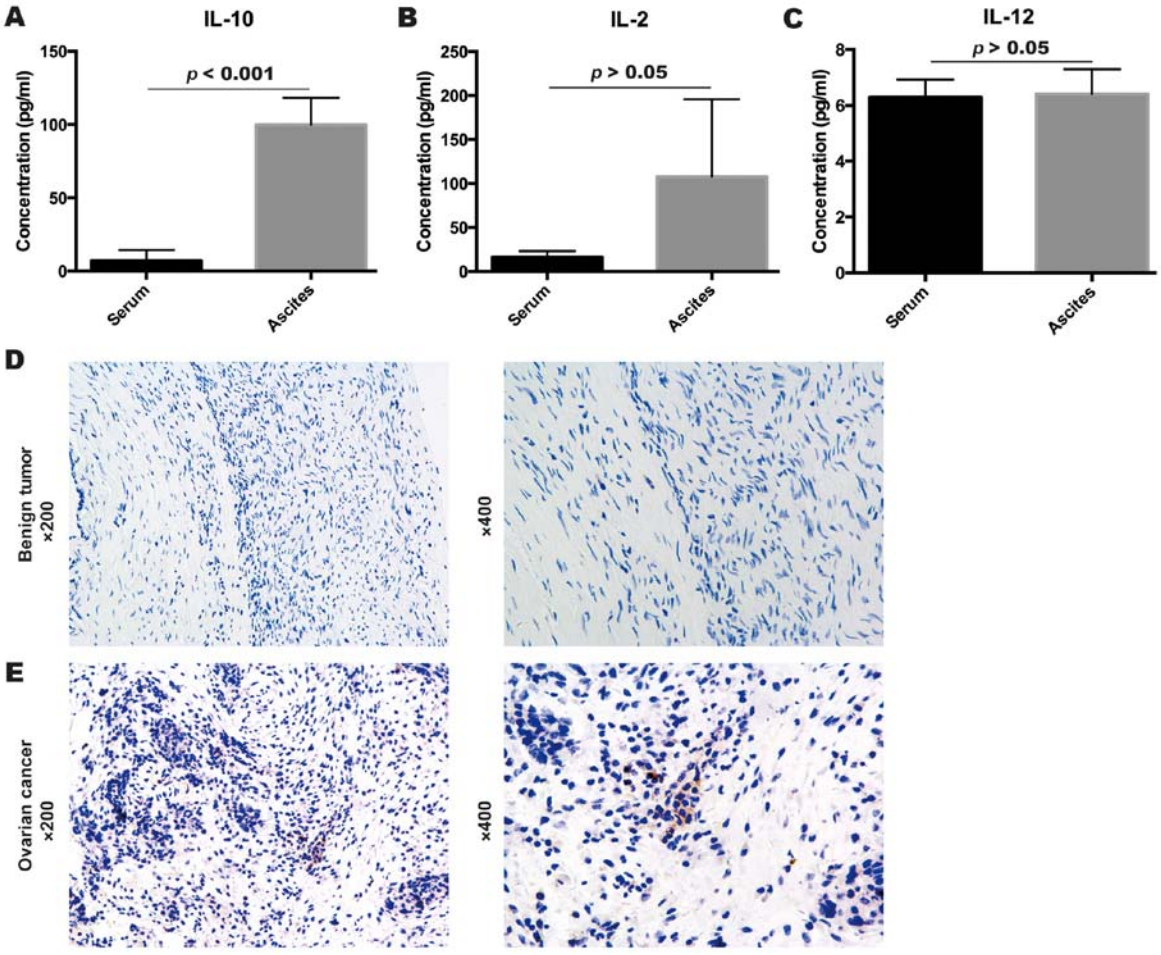

Figure 2. Expression levels of IL-10, IL-2 and IL-12 in EOC patients and healthy subjects by ELISA assays and the IL-10 expression in the EOC and the benign ovarian tumor samples by immunohistochemistry. (A) Inflammatory cytokine IL-10 expression level was significantly higher in the ascites of the EOC patients than that in the blood serum of the healthy subjects. (B and C) There were no significant differences in IL-2 and IL-12 between the ascites of the EOC patients and the serum of the healthy subjects. (D) IL-10 was stained in the benign ovarian tumor tissues by immunohistochemistry. (E) IL-10 was stained in the EOC samples; left, at a magnification of x200; right, at a magnification of x400. IL-10, interleukin-10; EOC, epithelial ovarian cancer.

cells and TAMs in the EOC and benign ovarian tumors. Treg cells were identified as $CD4^+$ and $Foxp3^+$. The frequency of Treg cells was confirmed to be higher in the EOC (4.26±2.19) than that in the benign ovarian tumors (0.53±0.98, p<0.001, Mann-Whitney U test) (Fig. 1C). TAMs were identified as $CD68^+$. The frequency of TAMs was also confirmed to be higher in the EOC (10.79±13.26) than that in the benign ovarian tumors (0.56±1.25, p<0.001, Mann-Whitney U test) (Fig. 1F). Moreover, EOC patients with high frequency of Treg cells exhibited a significantly shorter overall survival time compared to those with low frequency of Treg cells (p=0.045, Kaplan-Meier curves) (Fig. 1G). Thus, these results demonstrated that the distribution of Treg cells and TAMs was different in the EOC and benign ovarian tumors.

Inflammatory cytokines in ascites and serum samples. Expression levels of inflammatory cytokines IL-2, IL-10 and IL-12 in ascites samples of 16 EOC patients and blood serum samples of 7 healthy subjects were detected by ELISA assays. As shown in Fig. 2, the expression level of IL-10 was significantly higher in the ascites of EOC patients (99.81±71.67 pg/ml) than that in the blood serum of the healthy subjects (p<0.001, 7.23±7.26 pg/ml, Levene's test) (Fig. 2A). However, IL-2 and IL-12 expression levels had no significant difference between the ascites of EOC patients and the serum of the healthy subjects (p>0.05, IL-2, 107.76±351.55 pg/ml for the ascites vs. 16.38±6.98 pg/ml for the serum, Mann-Whitney U test; p=0.35, IL-12, 6.41±3.55 pg/ml for the ascites vs. 6.30±1.68 pg/ml for the serum, Mann-Whitney U test) (Fig. 2B and C).

Expression of IL-10 in EOC and benign ovarian tumor samples. To investigate the IL-10 expression in benign ovarian tumor samples as well as EOC samples, the expression of IL-10 was evaluated by immunohistochemistry. As shown in Fig. 2, compared to the negative expression of IL-10 in the benign ovarian tumor samples (Fig. 2D), the EOC samples had strong staining for IL-10 expression (Fig. 2E). Thus, we supposed that IL-10 may play an important role in EOC progression.

TAMs increase the frequency of Treg cells by IL-10. TAMs reportedly make up to 70% of immune cells in the EOC microenvironment (18) and one feature of TAMs is to secrete IL-10 (19). We induced monocytes into macrophages (M2, like TAMs) by M-CSF and IL-4 (Fig. 3). The M1 macrophage (M-CSF + LPS + IFN-r) was used as the M1

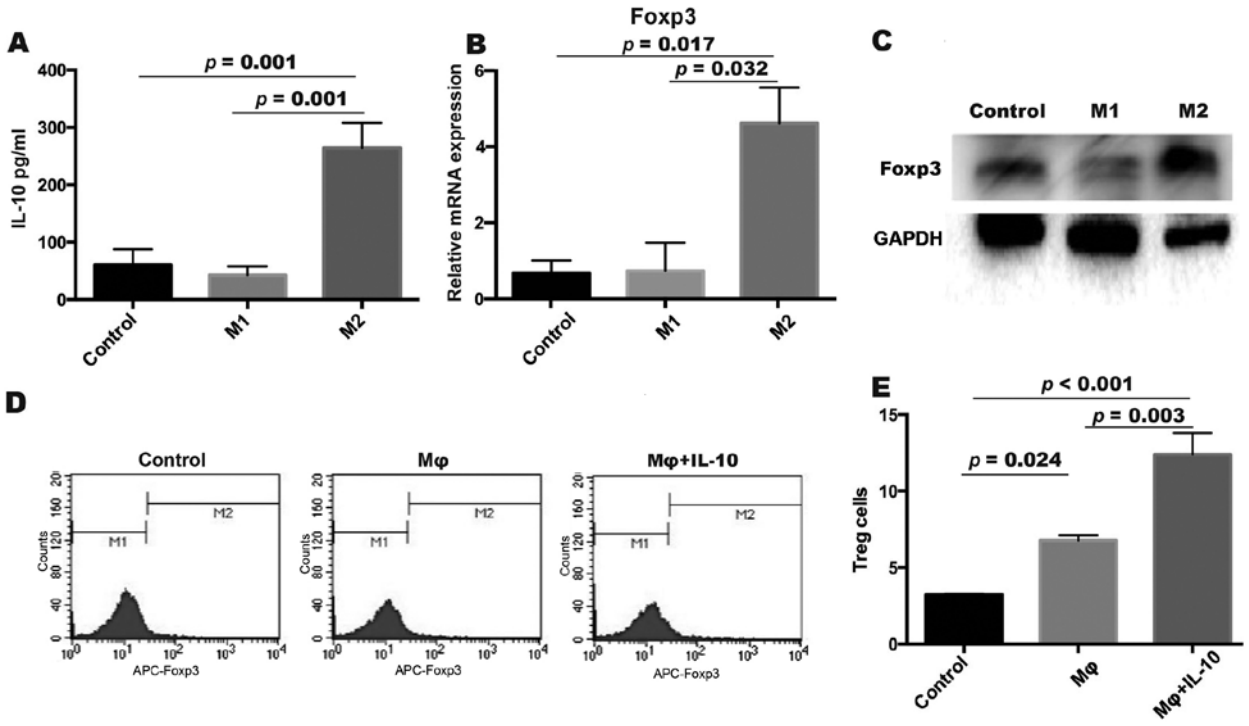

Figure 3. Expression of Treg cells is induced by IL-10 derived from TAMs via Foxp3 upregulation. (A) IL-10 was detected in the co-cultured supernatants. (B) The Foxp3 expression of CD4+ T cells in the control, M1 and M2 group was detected by real-time PCR. (C) The Foxp3 expression of CD4+ T cells in the aforementioned three groups was detected by western blotting. (D) The proportion of Foxp3+ Treg cells was detected in the control, Mφ and Mφ + IL-10 group by flow cytometric analysis. (E) The frequency of Tregs was higher in the Mφ + IL-10 group when compared to the other two groups. IL-10, interleukin-10; Tregs, regulatory T cells; TAMs, tumor-associated macrophages.

group. CD4+ T cells were co-cultured with the M1/M2 group. We then detected the IL-10 released in the co-cultured supernatants, and the results indicated that IL-10 in the M2 group (264.05±75.91 pg/ml) was significantly higher than that in the other groups (p=0.001 both for the M1 and control groups, control: 60.89±46.55 pg/ml; M1: 42.71±26.09 pg/ml; LSD test) (Fig. 3A). Next, we detected the expression of Foxp3 mRNA in CD4+ T cells after being cultured with M1/M2 macrophages using real-time PCR. We found that the expression of Foxp3 was higher in the M2 group (4.62±1.63) compared to the M1 group (0.74±1.28, p=0.032, Levene's test) and the control group (0.67±0.58, p=0.017, Levene's test) (Fig. 3B). The same result was also demonstrated by western blotting (Fig. 3C). These results suggested that IL-10 could upregulate Foxp3 expression to induce Treg cells during T differentiation.

Interleukin-10 induces the upregulation of Treg cells. To investigate whether IL-10 plays a significant role in the differentiation of Treg cells we next cultured CD4+ T cells with macrophages (Mφ group) and macrophages with IL-10 (Mφ + IL-10 group) for 3 days. After being co-cultured with macrophages and IL-10, the proportion of Foxp3+ Treg cells was detected by flow cytometric analysis (Fig. 3D). The frequency of Treg cells was higher in the Mφ + IL-10 group (12.39±2.45) when compared to the Mφ group (6.79±0.58, p=0.003, LSD test) and the control group (3.22±0.12, p<0.001, LSD test) (Fig. 3E). The results showed that IL-10 could increase the frequency of Treg cells.

Effect of IL-10 and CD4+ T cells on the growth of EOC tumors in vivo. To investigate the influence of IL-10 and CD4+ T cells on EOC development, we established a subcutaneous nude mouse model to observe EOC tumor growth (Fig. 4). The tumor weight (g) of the SKOV3-Luc + CD4+ T cells + IL-10 group (0.88±0.06 g) was much higher than that of the SKOV3-Luc + CD4+ T cell group (p=0.024, LSD test, 0.72±0.02 g) and the SKOV3 group (p=0.041, LSD test, 0.74±0.03 g) (Fig. 4A). Moreover, the tumor volume (mm^3) of the SKOV3-Luc + CD4+ T + IL-10 group (924.26±263.80 mm^3) was also higher than that of the SKOV3-Luc + CD4+ T cell group (p=0.032, LSD test, 645.96±41.17 mm^3) and the SKOV3 group (p=0.033, LSD test, 649.40±14.02 mm^3) on the 4th week (Fig. 4B). Furthermore, after injecting SKOV3-Luc cells and CD4+ T cells with or without IL-10, we used a luminescence imaging system to observe tumor growth. The SKOV3-Luc + CD4+ T cells + IL-10 group [$3.08x10^{10}$±$8.94×10^9$ photons (ph)/sec] showed an enhancement of tumor total flux (ph/sec) on the 4th week, compared to the SKOV3-Luc + CD4+ T cell group (p=0.043, LSD test, $1.28×10^{10}$±$2.38×10^9$ ph/sec) and the SKOV3 group (p=0.012, LSD test, $6.65×10^9$±$1.51×10^9$ ph/sec) (Fig. 4C and D).

Discussion

EOC contains malignant cells as well as a number of TAMs. Different macrophages perform different functions. Therefore, it is known that monocytes may be recruited by the tumor microenvironment and acquire an M2 macrophage phenotype, which is different from an M1 macrophage phenotype. M2 macrophages play a key role in EOC growth, progression and peritoneal metastasis through the secretion of cytokines and growth factors, thereby contributing to tumor angiogenesis

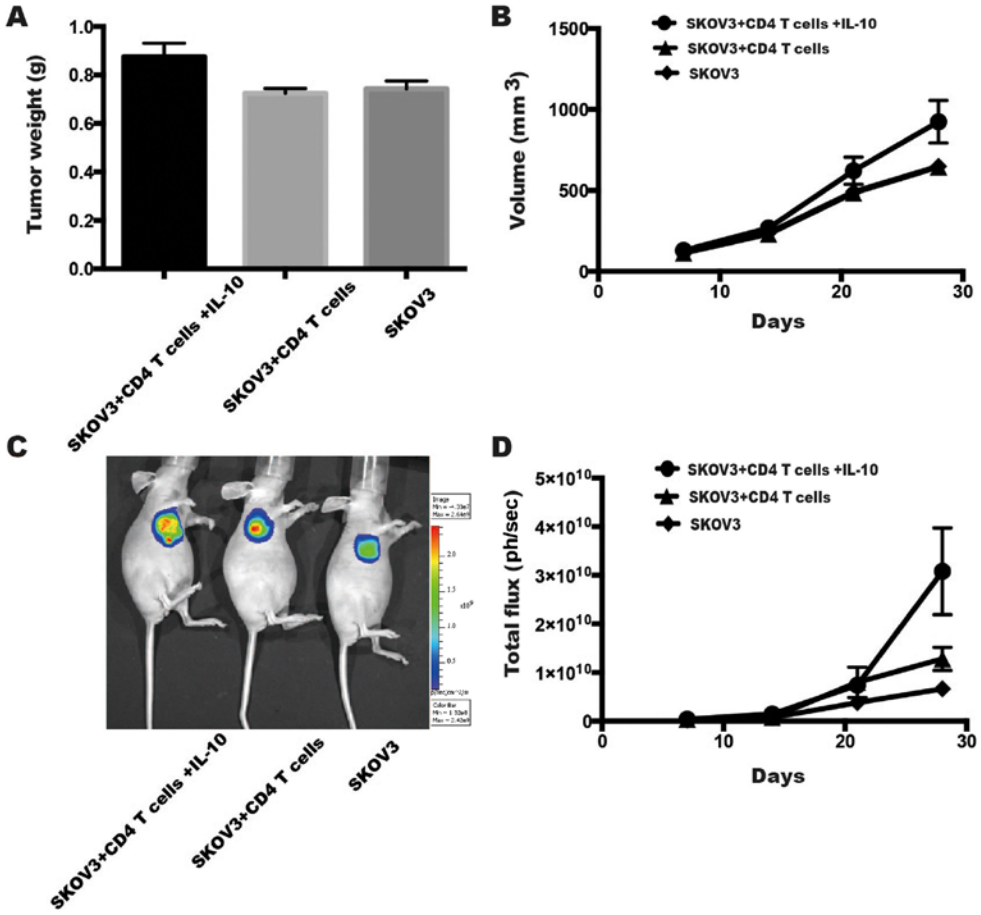

Figure 4. Effect of IL-10 and $CD4^+$ T cells on the growth of EOC tumors *in vivo*. (A and B) The tumor weight (g) and volume (mm^3) of the SKOV3-Luc + $CD4^+$ T cells + IL-10 group were much higher than that of the SKOV3-Luc cells + $CD4^+$ T cells and the SKOV3 group. (C and D) Bioluminescence imaging system was used to observe tumor growth. The SKOV3-Luc + $CD4^+$ T cells + IL-10 group led to an enhancement in tumor total flux [photons(ph)/sec] compared to the other two groups. IL-10, interleukin-10; EOC, epithelial ovarian cancer.

and immunosuppression (20). The poor prognosis of carcinomas, which includes bladder, cervical and breast cancer, may be due to the macrophage infiltration in tumors (21-23). Macrophages are a key source of critical cytokine IL-10 (24). We therefore hypothesized that IL-10 secreted by TAMs may increase the frequency of Treg cells. In this study, we investigated the frequency of TAMs and Treg cells in human EOC and benign ovarian tumors. TAMs and Treg cells were observed in patients with EOC more than in subjects with benign ovarian tumors. In addition, the expression of IL-10 was also observed to be higher in EOC patients. IL-10 is an immunosuppressive cytokine, which is secreted by TAMs. It is known that IL-10 contributes to the suppression of antitumor activity in the tumor microenvironment. Our results also identified that the expression level of IL-10 was significantly higher in the ascites of EOC patients than that in the blood serum of the healthy subjects. Thus, these data demonstrated that the soluble factor IL-10 derived from TAMs was responsible for the EOC activity in the tumor microenvironment and may promote EOC progression.

Treg cells are known to play a vital role in suppressing antitumor functions. Some studies have shown that during the early stage of differentiation, Treg cells may require TGF-β, which is secreted by TAMs, to hamper immunoregulatory responses (15). Other studies have demonstrated that Treg cells may mediate immunosuppression by IL-10 in the tumor microenvironment (25-27). With respect to Treg cells, Islas-Vazquez *et al* proposed that IL-10 may participate in the differentiation process of Treg cells in lung adenocarcinoma (28). Our results indicated that IL-10 secreted by TAMs may participate in the differentiation of T cells during development. Thus, IL-10 cytokine that is increased in the tumor microenvironment in EOC patients promotes the presence of Treg cells and increases in Treg cells is associated with EOC progression, peritoneal metastasis, which eventually results in poor prognosis.

Treg cells are a subpopulation of T cells with immunosuppressive response and express transcription factor Foxp3. It is reported that Foxp3 plays an important role in regulatory T-cell function (17). Nevertheless, Foxp3 has its limited value in the isolation of Treg cells in function assays, because it is a nuclear protein. Our data demonstrated that the Foxp3 expression of Treg cells was higher in the M2 group. Thus, IL-10 detected in this study may correlate with Foxp3 expression in Treg cells and be involved in immunosuppression in EOC. This knowledge may lead to the development of immunotherapy that inhibits the suppressive activity mediated by IL-10 from TAMs. Further research is required to explore this possibility.

In conclusion, our results helped clarify that IL-10 mediated interaction between TAMs and Treg cells, suggesting that

the targeting of TAMs and their cytokines may be potential targets for novel immunotherapy in the treatment of ovarian cancer. This conclusion may have important clinical value for the diagnosis, therapy and prognosis of EOC patients.

Acknowledgements

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