International Immunopharmacology 68 (2019) 81–87

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Follicular regulatory T cells infiltrated the ovarian carcinoma and resulted in CD8 T cell dysfunction dependent on IL-10 pathway

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ARTICLE INFO

Keywords:
Follicular regulatory T cells
IL-10
CD8 T cells
Ovarian cancer

ABSTRACT

A high Treg/CD8 T cell ratio in ovarian carcinoma was negatively associated with the prognosis of the patients. The human follicular regulatory T (Tfr) cells are a newly characterized subset of Treg cells with features of both follicular helper T (Tfh) cells ($CXCR5^+$) and canonical Treg cells ($CD25^+Foxp3^+$). The role of Tfr cells in ovarian cancer is yet unclear. We found that in peripheral blood, the ovarian cancer patients presented significantly higher levels of both $CD4^+CD25^+CD127^-CXCR5^+$ T cells and $CD4^+CD25^+CD127^-CXCR5^+Foxp3^+$ T cells than the healthy controls. In resected tumor samples, Tfr cells represented a much greater percentage of lymphocytes than in peripheral blood. Interestingly, the circulating Tfr cells from ovarian cancer patients presented significantly higher TGFB1 and IL10 expression than their counterparts in healthy controls directly ex vivo, and significantly higher IL10 after stimulation. The tumor-infiltrating Tfr cells presented further upregulated expression of TGFB1 and IL10. In addition, the levels of TGFB1 and IL10 expression by Tfr cells negatively associated with the expression of IFNG in tumor-infiltrating CD8 T cells. In an in vitro CD8 T cell/Tfr cell coculture system, we found that Tfr cells could significantly suppress the activation of CD8 T cells, in a manner that was dependent on IL-10 and probably on TGF-β. Overall, our study found that Tfr cells could suppress CD8 T cells, and in ovarian cancer patients, the Tfr cells were increased in both frequency and function.

1. Introduction

Globally, primary ovarian cancer represents approximately 1.7% of all cancers in women [1]. Although a number of treatment options are available, this disease remains the most lethal among all cancers unique to women. The majority of ovarian cancers at diagnosis are at advanced stages (III and IV) with fewer treatment options. Moreover, many subtypes of ovarian cancer with heterogeneous characteristics are identified, further complicating the treatment. Currently, only about 15% to 20% of women with ovarian cancer are able to survive without recurrence ten years post-diagnosis [2,3]. Better understanding of the pathogenesis and progression of ovarian cancer is urgently needed to improve the outcome of the disease.

The immune system is involved in many aspects of ovarian cancer. It is thought that the rupture of the ovulating follicle during ovulation may cause an inflammatory response, which increases the risk of malignant transformation over time [4]. On the other hand, antitumor immune responses are considered beneficial in ovarian cancer patients. Previous studies demonstrated that the number of total CD3 T cells and cytotoxic CD8 T cells that infiltrate the tumor positively correlated with survival, possibly due to immune cell recruitment, activation and tumor cell elimination mediated by chemokines, IFN-γ, and granzymes, respectively [5–7]. The infiltration of CD20-expressing (B cell marker) lymphocytes was also associated with favorable outcomes in ovarian cancer [8]. These $CD20^+$ B cells tended to colocalize with CD8 T cells and might have increased the cytotoxic activity of CD8 T cells by providing antigen presentation. At the same time, the tolerance-inducing regulatory T (Treg) cells in ovarian cancer associated with poorer prognosis [6,9,10]. It is thought that Treg cells help establish a suppressive microenvironment in the tumor by expressing the inhibitory molecule CTLA-4 and the suppressive cytokines IL-10 and TGF-β [11]. IL-10 and TGF-β may also directly contribute to cancer pathogenesis by promoting tumor cell growth and eliciting metastasis [12,13].

T follicular regulatory (Tfr) cells are a recently characterized Treg subset with both T follicular helper (Tfh) cell features, such as CXCR5 and ICOS expression, and Treg cell features, such as CTLA-4 and Foxp3 expression [14]. The expression of CXCR5 enables Tfr cells to access B cell-rich areas and modulate both Tfh cell and B cell responses during inflammation [14,15]. Depletion of Tfr cells in mice resulted in higher serum antibody titer and higher numbers of B cells [16,17]. In vitro experiments also demonstrated that human Tfr cells could suppress antibody production [18]. Spontaneous autoimmunity can arise upon

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https://doi.org/10.1016/j.intimp.2018.12.051
Received 1 October 2018; Received in revised form 13 December 2018; Accepted 23 December 2018
Available online 04 January 2019
1567-5769/

Tfr-depletion in mouse Sjögren's disease model [19], demonstrating that Tfr cells possess critical roles in suppressing inflammation. In acute virus infections, Tfr cells were reduced in frequency, thus allowing the development of antiviral antibodies, while in chronic HIV, HBV, and HCV infections, an enrichment of Tfr cells was found [20,21]. The function of Tfr cells in cancer has not been extensively examined, but many studies have shown that the frequency of Tfr cells was altered in malignancies. In non-small cell lung cancer, Tfr frequency was elevated in blood [22], while in diffuse large B cell lymphoma, Tfr cells were enriched in the lymph nodes and their frequency was reduced in higher stages [23]. Also, in follicular lymphoma, Tfr cells accumulated in the tumor microenvironment [24].

In this study, we sought to characterize Tfr cells in ovarian cancer patients. The frequency of Tfr cells in circulation and in the tumor and the functional potency of Tfr cells have been examined, compared, and contrasted in healthy controls and ovarian cancer patients.

2. Materials and methods

2.1. Study participants

Cancer patients were recruited from women with primary presentation of ovarian carcinoma, who were newly diagnosed, previous untreated, and had no previous history of any other form of malignancy. Healthy subjects were recruited from age- and BMI-matched women with no history of malignancy. The detailed characteristics of the patients and controls are displayed in Table 1. In addition, the patients and controls were not experiencing ongoing acute or chronic infections, were not obese, and had no evidence of previous or present autoimmune disorders. A total of 20 patients and 20 healthy volunteers provided peripheral blood samples, and 16 of the 20 cancer patients also provided resected tumor samples. The ethics review board of the Third Affiliated Hospital of Xinjiang Medical University approved this study. All participants provided written informed consent.

2.2. Cell collection

Venous blood was collected into citrate-containing tubes. Peripheral blood mononuclear cells were harvested from blood using centrifugation across a Ficoll-PLUS (GE Healthcare) gradient. Tumor was rinsed in 10× Penicillin-Streptomycin (Thermo Fisher Scientific) in cold PBS, minced to small pieces, and incubated in dispase II (2.4 U/mL) solution (Sigma) for 30 min at 37 °C. Undigested clumps were filtered across a 70-μm sterile mesh, and the tumor-infiltrating lymphocytes were collected using Ficoll-PLUS gradient centrifugation.

2.3. Flow cytometry

Circulating or tumor-infiltrating lymphocytes were incubated with anti-human CD3, CD4, CD25, and CD127 antibodies (BioLegend) and the Fixable viability dye eFluor 780 (Thermo Fisher Scientific) for 30 min at 4 °C. After washing, the cells were incubated in Foxp3 Fixation/Permeabilization buffer (Thermo Fisher Scientific) for 5 min, washed again, and incubated with anti-human Foxp3 antibody or isotype control antibody (Thermo Fisher Scientific) for 30 min at 4 °C. The Tfr cell frequency was evaluated in the FACSCanto system (BD Biosciences).

Table 1
Characteristics of the healthy controls and ovarian cancer patients.

	Control	Patient	
		Stage I/II	Stage III/IV
N	20	6	14
Age (years, mean ± SD)	44.5 ± 6.1	47.3 ± 8.1	46.2 ± 7.7
Menopausal status (N, pre/peri/post)	10/5/5	3/2/1	6/3/5

2.4. Tfr and CD8 T cell isolation

CD4 T cells and CD8 T cells were isolated using the Human CD4 T Cell Enrichment Kit and the Human CD8 T Cell Enrichment Kit (Stemcell), respectively. The Tfr cells were then isolated from purified CD4 T cells by $CD4^+CD25^+CD127^-CXCR5^+$ staining in the FACSAria system (BD Biosciences). For Tfr stimulation, 50 ng/mL PMA and 1 μg/mL ionomycin (Calbiochem) were added for 6 h. The TGFB1 and IL10 gene expressions were then evaluated using TaqMan assays with commercial primer sets Hs00961622_m1 and Hs00998133_m1 (Thermo Fisher Scientific), respectively.

2.5. Tfr cell/CD8 T cell coculture

CD8 T cells were placed in a 96-well flat-bottom anti-CD3/CD28-coated plate at 5×10^4 cells per well. An equal number of sorted Tfr cells were added. In some experiments, anti-human IL-10 (clone 9D7; Mabtech), anti-human TGF-β antibody (clone 9016; R&D Systems), or the relevant isotype control antibodies were added at 10 μg/mL. The cells were incubated for 72 h at 37 °C in a CO_2 incubator, and then transferred to a 96-well V-bottom plate. Supernatant was collected by centrifugation and the IFN-γ concentration was examined using the Human IFN gamma ELISA Kit (Abcam) following the instructions from the manufacturer. The CD8 T cells were purified from the coculture using the Human CD8 T Cell Enrichment Kit, and the IFNG gene transcription was examined using the TaqMan assay with primer set Hs00989291_m1 (Thermo Fisher Scientific).

2.6. Statistical analysis

Mean ± eSD was reported where applicable. Comparisons between two unmatched or matched groups were examined using unpaired or paired *t*-tests, respectively. Comparisons between three matched groups were examined using RM 1-way ANOVA followed by Dunnett's test. Correlation between two parameters was evaluated using Pearson correlation test. Two-tailed P smaller than 0.05 was considered to be statistically significant.

3. Results

3.1. Tfr frequency in ovarian cancer patients and healthy volunteers

Human Tfr cells have been sorted as $CD4^+CD25^+CD127^-CXCR5^+$ T cells [18], and should also express the Treg transcription factor Foxp3. In the first part of the study, we examined the frequency of both $CD4^+CD25^+CD127^-CXCR5^+$ T cells and $CD4^+CD25^+CD127^-CXCR5^+Foxp3^+$ T cells in the peripheral blood using flow cytometry (Fig. 1a). $CD4^+CD25^+CD127^-CXCR5^+$ T cells represented 0.84% ± 0.35% (mean ± SD) of total circulating lymphocytes in healthy subjects and 1.13% ± 0.45% of total circulating lymphocytes in ovarian cancer patients (Fig. 1b). The vast majority (> 80% in all study subjects with median values of 92% in healthy subjects and 93% in ovarian cancer patients) of $CD4^+CD25^+CD127^-CXCR5^+$ T cells also expressed Foxp3, and the frequency of $CD4^+CD25^+CD127^-CXCR5^+Foxp3^+$ T cells in total lymphocytes was 0.76% ± 0.31% in healthy subjects and 1.05% ± 0.42% in patients (Fig. 1c). Both cell types were significantly higher in ovarian cancer patients than in healthy controls.

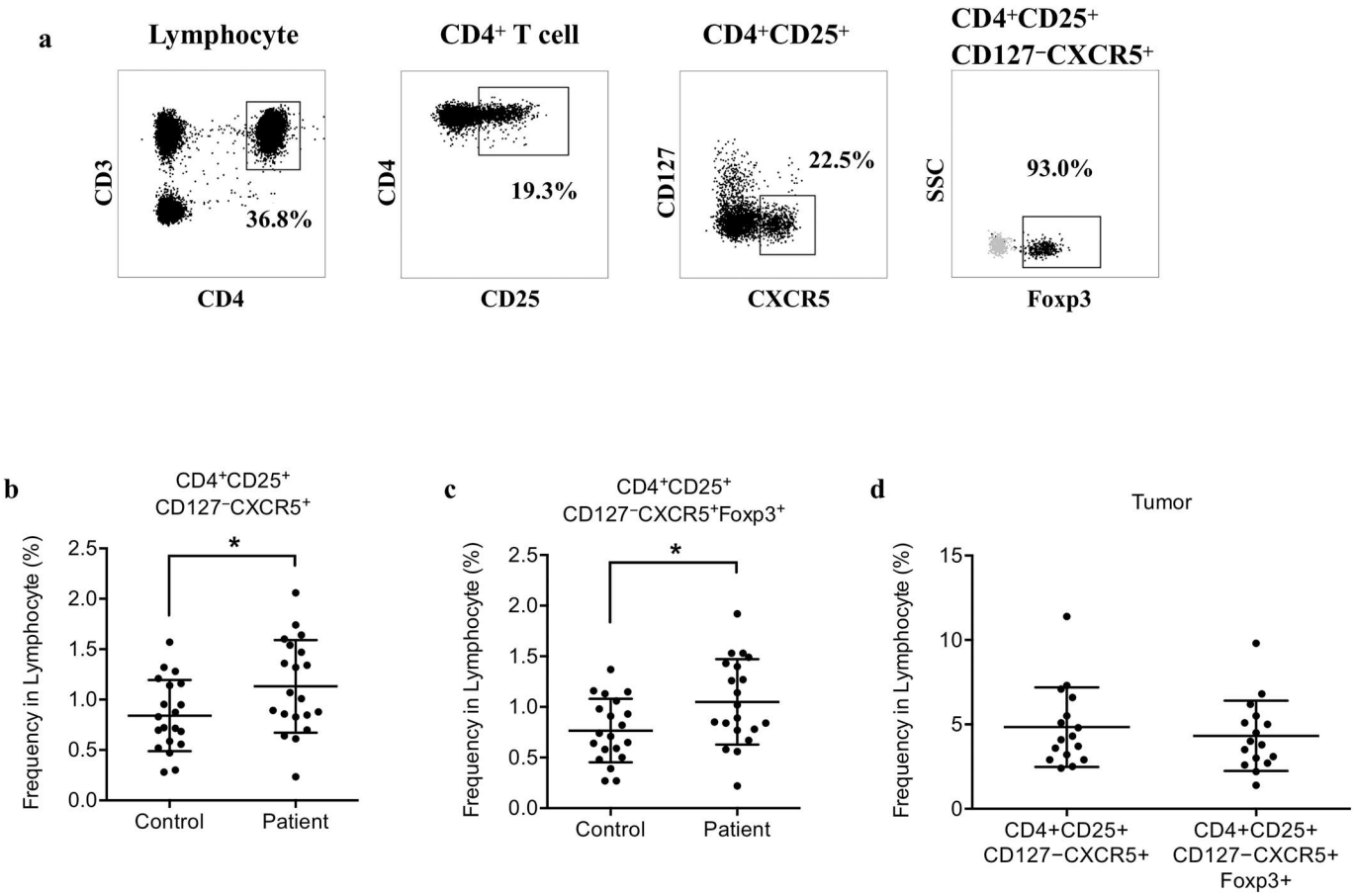

Fig. 1. Tfr cell frequencies in ovarian cancer patients and healthy volunteers.
a. Sample identification of $CD4^+CD25^+CD127^-CXCR5^+$ T cells and $CD4^+CD25^+CD127^-CXCR5^+Foxp3^+$ T cells using flow cytometry. The Foxp3-positive gating was confirmed by an isotype control antibody (grey background) against the anti-Foxp3 antibody (black foreground). b. The frequency of $CD4^+CD25^+CD127^-CXCR5^+$ T cells in circulating lymphocytes. c. The frequency of $CD4^+CD25^+CD127^-CXCR5^+Foxp3^+$ T cells in circulating lymphocytes. d. The frequency of $CD4^+CD25^+CD127^-CXCR5^+$ T cells and $CD4^+CD25^+CD127^-CXCR5^+Foxp3^+$ T cells in tumor-infiltrating lymphocytes. Unpaired *t*-test with unequal variances. *$P < 0.05$.

3.2. Tfr cells in resected tumors from ovarian cancer patients

In 16 ovarian cancer patients whose resected tumor samples were available, we examined the frequency of tumor-infiltrating Tfr cells. We found that $CD4^+CD25^+CD127^-CXCR5^+$ T cells represented 4.80% ± 2.56% of total tumor-infiltrating lymphocytes, most of which were also $CD4^+CD25^+CD127^-CXCR5^+Foxp3^+$ T cells, representing 4.23% ± 2.24% of total tumor-infiltrating lymphocytes (Fig. 1d).

3.3. Cytokine expression by circulating and tumor-infiltrating Tfr cells

Canonical $Foxp3^+$ Treg cells express TGF-β and IL-10 upon stimulation, and these cytokines are critical for establishing peripheral tolerance and suppress inflammation [25]. Here, the $CD4^+CD25^+CD127^-CXCR5^+$ T cells were sorted from circulating lymphocytes, and the expressions of TGF-β and IL-10 was examined through gene transcription and protein secretion. Directly ex vivo, the protein secretion was undetectable, but the TGF-β (TGFB1) and IL-10 (IL10) gene transcription could be detected, and the $CD4^+CD25^+CD127^-CXCR5^+$ T cells from ovarian cancer patients presented significantly higher levels of TGFB1 and IL10 transcription than those cells from control volunteers (Fig. 2a and b). To stimulate cytokine expression, the $CD4^+CD25^+CD127^-CXCR5^+$ T cells were stimulated with PMA and ionomycin. After stimulation, the $CD4^+CD25^+CD127^-CXCR5^+$ T cells from ovarian cancer patients presented comparable level of TGFB1 transcription with that from healthy subjects (Fig. 2c), but significantly higher level of IL10 transcription than that from healthy subjects (Fig. 2d). A similar trend was observed in the secretion of TGF-β and IL-10 proteins by $CD4^+CD25^+CD127^-CXCR5^+$ T cells from patients and controls (Fig. 2e and f).

We also examined the TGF-β and IL-10 expression by $CD4^+CD25^+CD127^-CXCR5^+$ T cells from resected ovarian cancer patients. Due to the fact that the quantity of tumor-infiltrating cells was limited, we only examined the gene transcription, and that of both TGFB1 and IL10 could be detected in $CD4^+CD25^+CD127^-CXCR5^+$ T cells directly ex vivo (Fig. 3a). Compared to the expression of TGFB1 and IL10 in circulating Tfr cells, the expression in tumor-infiltrating Tfr cells was significantly elevated.

3.4. TGFB1 and IL10 expression by Tfr cells negatively associated with CD8 T cell response in ovarian tumor

The IFNG gene transcription, a hallmark of CD8 T cell activation, was evaluated in the CD8 T cells from resected ovarian tumors. Interestingly, we found that the transcription levels of both TGFB1 and IL10 in tumor-infiltrating $CD4^+CD25^+CD127^-CXCR5^+$ T cells were negatively correlated with the transcription level of IFNG in tumor-infiltrating CD8 T cells (Fig. 3b and c).

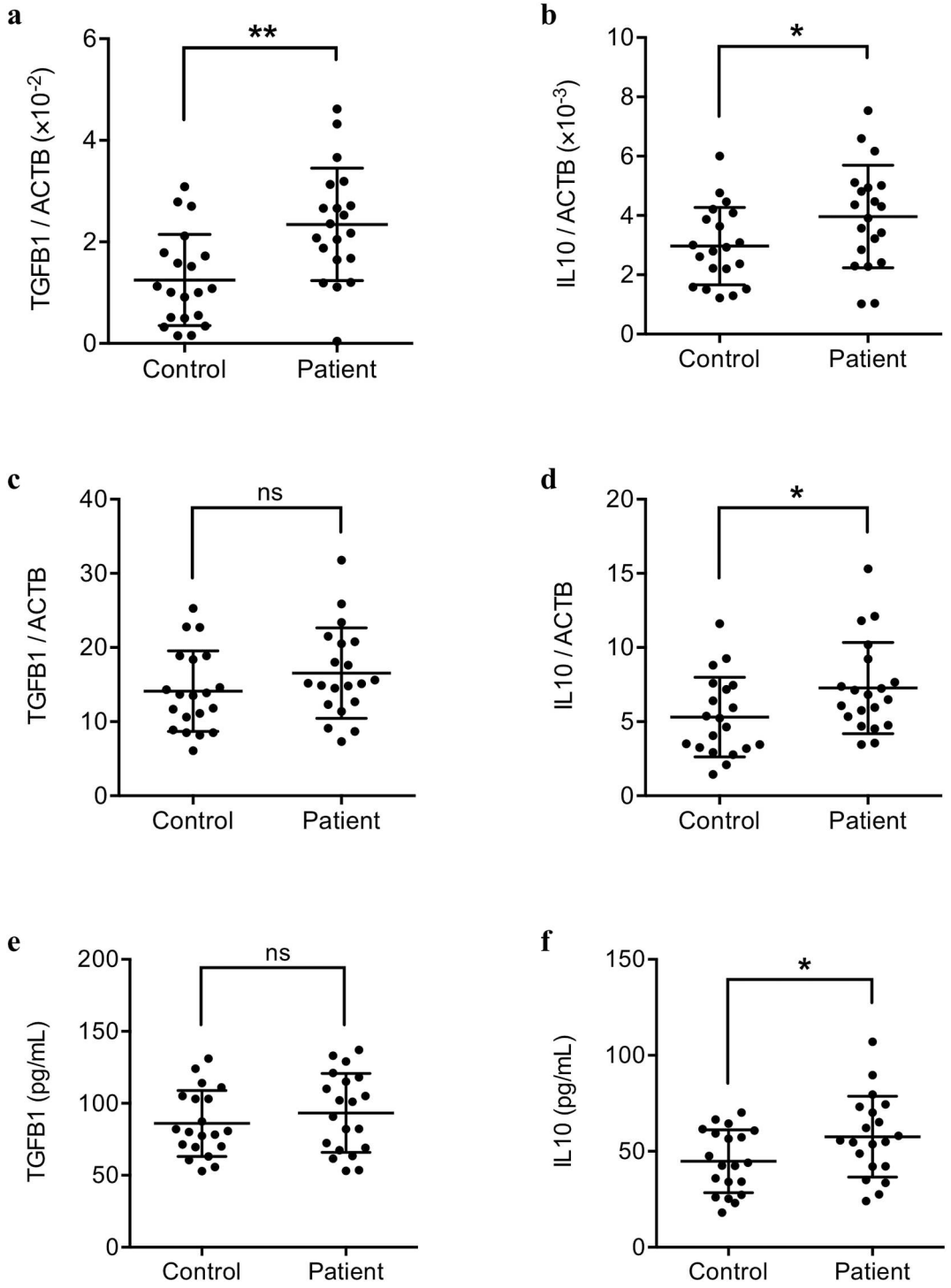

Fig. 2. TGF-β and IL-10 expression by Tfr cells.
a and b. $CD4^+CD25^+CD127^-CXCR5^+$ T cells were sorted from circulating lymphocytes and the transcription of (a) TGFB1 and (b) IL10 was examined directly ex vivo. c to f. $CD4^+CD25^+CD127^-CXCR5^+$ T cells were sorted from circulating lymphocytes and stimulated with PMA and ionomycin for 72 h. The cell was lysed and the transcription of (c) TGFB1 and (d) IL10 was examined by RT-PCR, while the secreted levels of (e) TGF-β and (f) IL-10 in the supernatant was examined by ELISA. Unpaired *t*-test with unequal variances. *$P < 0.05$. **$P < 0.01$. ns, not significant.

3.5. *Tfr suppressed CD8 T cell in vitro*

Next, we examined whether Tfr cells could directly suppress CD8 T cell activation, using a co-incubation setup with CD8 T cells and autologous circulating Tfr cells ($CD4^+CD25^+CD127^-CXCR5^+$ T cells) from blood lymphocytes of the patients. The expression of IFN-γ by CD8 T cells was then examined in the presence and absence of Tfr cells. The IFNG transcript levels in CD8 T cells were significantly lower in the presence of Tfr cells than in the absence of Tfr cells (Fig. 4a). The secreted IFN-γ level in the supernatant was also lower in the presence of Tfr cells than in the absence of Tfr cells (Fig. 4b).

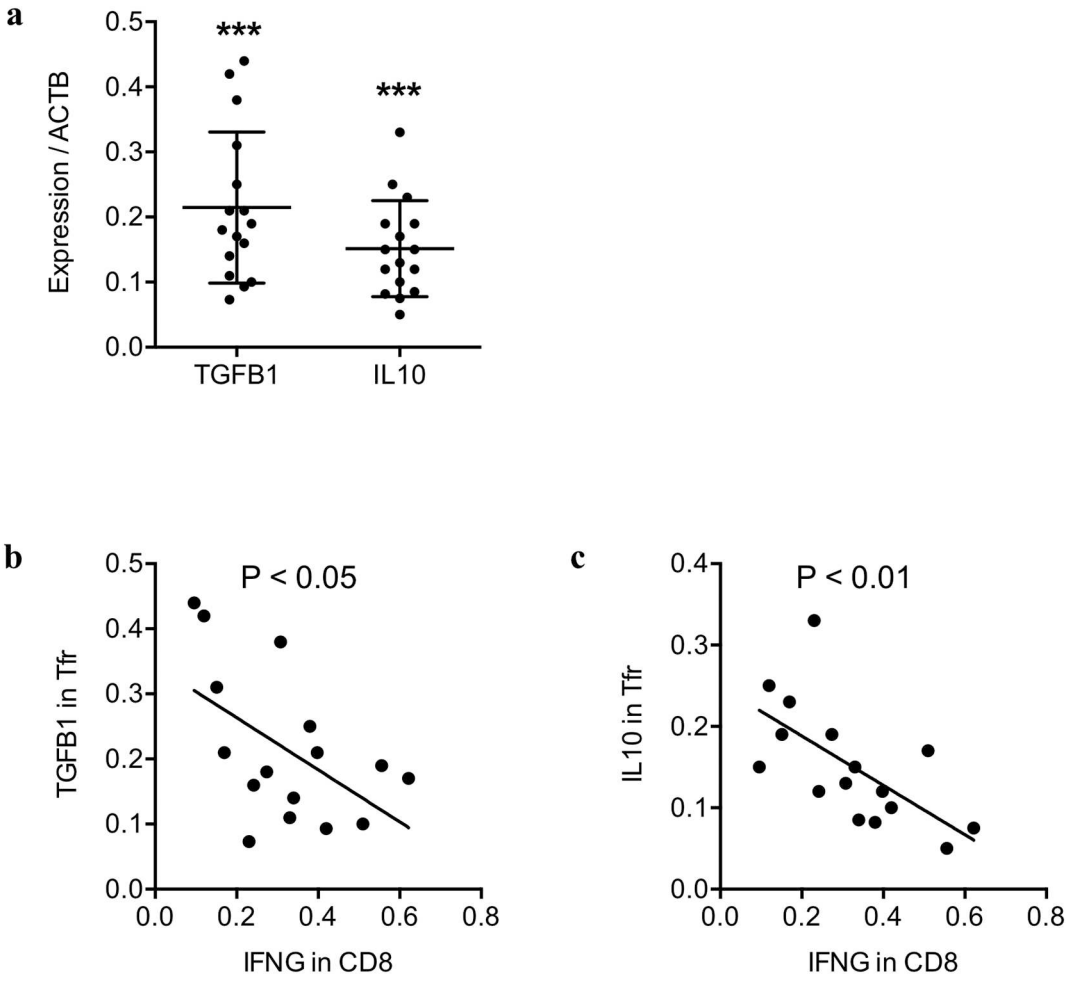

Fig. 3. TGFB1 and IL10 expression by tumor-infiltrating Tfr cells.
a. $CD4^+CD25^+CD127^-CXCR5^+$ T cells were sorted from tumor-infiltrating lymphocytes and the transcription of TGFB1 and IL10 was examined directly ex vivo. Results were compared with the corresponding results in circulating Tfr cells using unpaired *t*-test with unequal variances. ***P < 0.001. b and c. IFNG gene transcription was examined in tumor-infiltrating CD8 T cells and its correlation with (b) TGFB1 and (c) IL10 in tumor-infiltrating $CD4^+CD25^+CD127^-CXCR5^+$ T cells was examined using Pearson correlation test.

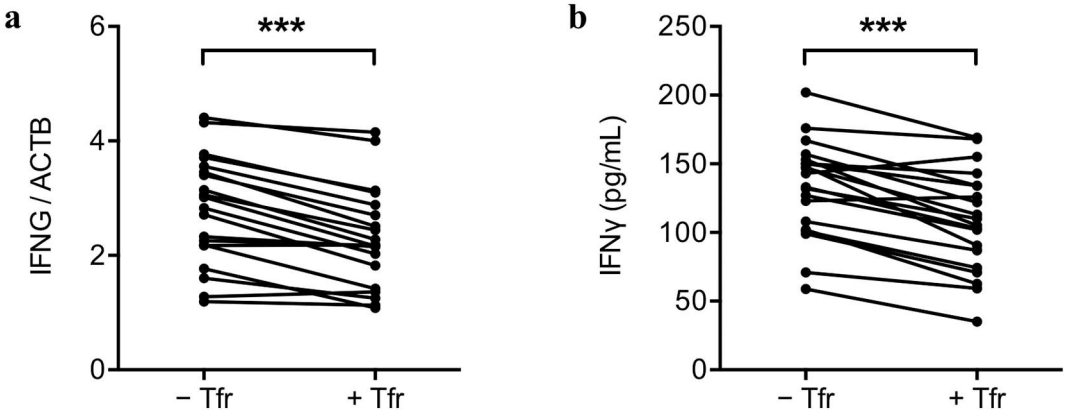

Fig. 4. IFN-γ by CD8 T cells in the absence or presence of Tfr cells.
CD8 T cells were incubated in the absence or presence of autologous $CD4^+CD25^+CD127^-CXCR5^+$ T cells, together with anti-CD3/CD28 stimulation. a. After 72 h, CD8 T cells were isolated from the coculture and the transcription of IFNG was examined by RT-PCR. b. The supernatant was harvested and the level of secreted IFN-γ was examined by ELISA. Paired *t*-test. ***P < 0.001.

3.6. Tfr-mediated suppression of CD8 T cells depended on IL-10 and TGF-β

To investigate whether Tfr cells mediated CD8 T cell suppression through the production of IL-10 and TGF-β, in the coculture of CD8 T cells and Tfr cells, we neutralized these cytokines using neutralizing antibodies. The IFN-γ gene transcription in CD8 T cells and protein secretion were then examined. IL-10 neutralization significantly elevated the expression of IFN-γ (Fig. 5a and b). TGF-β neutralization

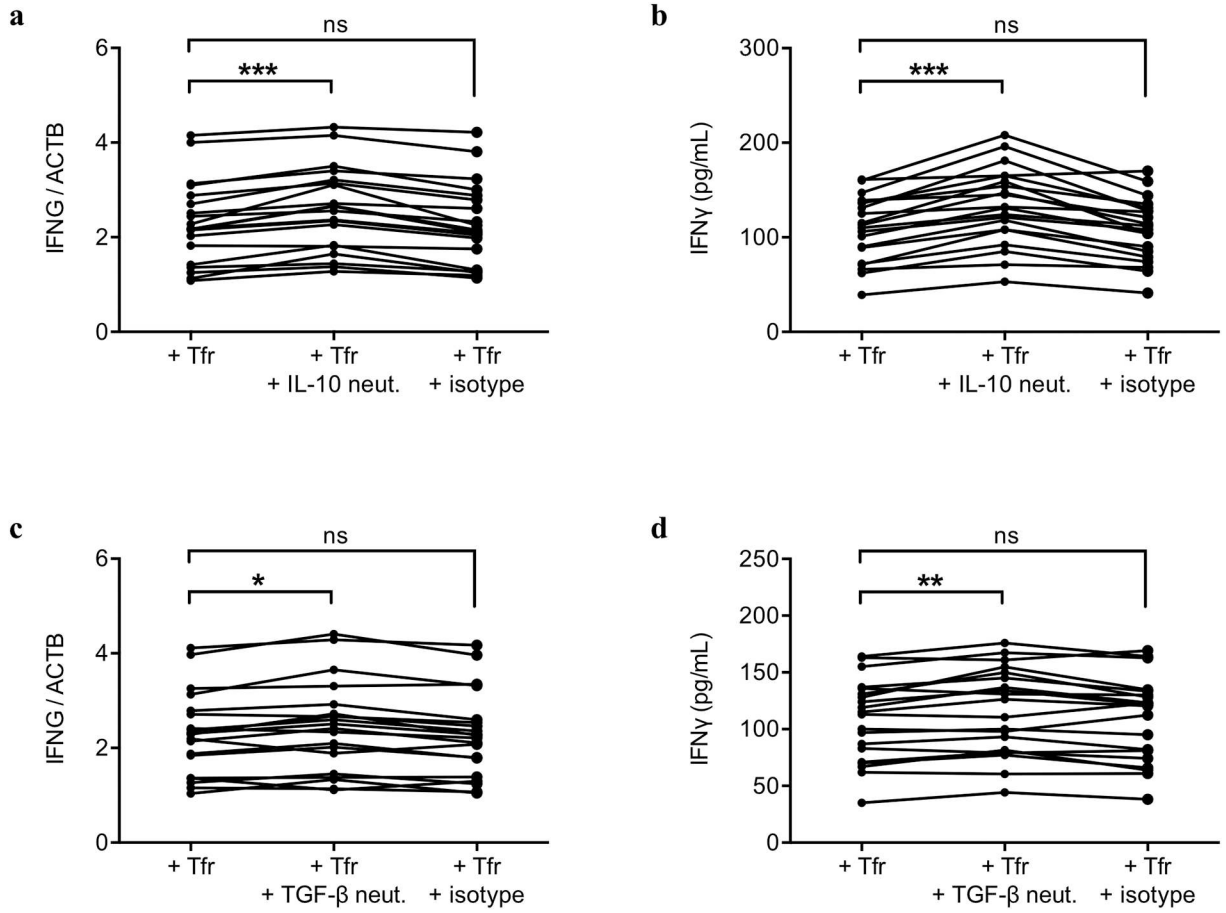

Fig. 5. Neutralization of IL-10 and TGF-β on the expression of IFN-γ by CD8 T cells.
a and b. CD8 T cells and $CD4^+CD25^+CD127^-CXCR5^+$ T cells were co-incubated in the presence of anti-CD3/CD28 stimulation for 72 h. IL-10 neutralizing antibody or isotype control antibody was added at 10 μg/mL when indicated. a. The transcription of IFNG was examined in isolated CD8 T cells, while b. the IFN-γ protein secretion was examined in the supernatant. c and d. CD8 T cells and $CD4^+CD25^+CD127^-CXCR5^+$ T cells were co-incubated in the presence of anti-CD3/CD28 stimulation for 72 h. TGF-β neutralizing antibody or isotype control antibody was added at 10 μg/mL when indicated. c. The transcription of IFNG was examined in isolated CD8 T cells, while d. the IFN-γ protein secretion was examined in the supernatant. RM 1-way ANOVA followed by Dunnett's test. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ns, not significant.

similarly increased the expression of IFN-γ in most patients (Fig. 5c and d), with a few exceptions.

4. Discussion

High Treg function is associated with poorer prognosis in cancer patients in general. Treg cells can suppress antitumor immune responses through multiple mechanisms, such as downregulating costimulatory signals on antigen-presenting cells and inhibit effector T cell activation via suppressive cytokines. In this study, we examined one specific subset, the CXCR5-expressing Tfr cells. This cell subset is best characterized as a suppressor of Tfh and B cell differentiation and function [15,26]. Here, we discovered that compared to healthy controls, the ovarian cancer patients presented significantly higher level of circulating Tfr cells, together with higher TGFB1 and IL10 expression directly ex vivo, and higher IL-10 expression after PMA and ionomycin stimulation. In addition, Tfr cells were present in all the tumor samples we examined, and in tumors, this cell type represented a significantly higher percentage of lymphocytes than that in peripheral blood. Furthermore, we found that the levels of TGFB1 and IL10 transcription in tumor-infiltrating Tfr cells were negatively associated with the level of IFNG transcription in tumor-infiltrating CD8 T cells. This discovery prompted us to examine the effect of Tfr cells on CD8 T cells. Using an in vitro coculture system, we found that the Tfr cells significantly suppressed the expression of IFN-γ by autologous CD8 T cells, and this suppression was dependent on IL-10 and, in part, on TGF-β. Overall, our study found that aside from suppressing Tfh and B cell responses, Tfr cells could also suppress CD8 T cells, and in ovarian cancer patients, the Tfr cells were increased in both frequency and function.

Most of the studies regarding the function of Tfr cells have focused on their effects on Tfh cells, B cells, and antibody production, and it remains unclear how Tfr and Treg cells may be similar to different in suppressing T cell inflammation and modulating antigen-presenting cells. Given the fact that Tfr cells, but not conventional Treg cells, express CXCR5, we would hypothesize that Tfr cells might be more capable of modulating the antigen presenting function and cytokine secretion from B cells, but further experiments are needed. Also, in many studies, CXCR5 was not included in the staining panel, so it was unclear whether the tumor-infiltrating Treg cells included a Tfr subset. In a previous study by Cha et al. in diffuse large B cell lymphoma, Tfr cells and non-Tfr Treg cells both suppressed Tconv cell and CD8+ T cell proliferation, but Tfr cells were less capable of suppressing IFN-γ than Treg cells [23]. Further experiments using healthy and cancer animal models should be employed to confirm our finding in this study, and explore the similarity and differences between Tfr cells and Treg cells. In addition, the localization of Tfr cells in relationship with effector $CD8^+$ T cells, $CD4^+$ T cells, Treg cells, and B cells inside the tumor should be investigated using immunohistochemistry.

It remains unclear why Tfr cells were more enriched in the tumors than in circulating blood. In follicular lymphoma, mesenchymal stromal

cells in the tumor microenvironment can induce FOXP3 expression in Tfh cells, thus promoting the de novo generation of Tfr cells [24]. Also, it has been shown that tumor-associated macrophages (TAMs) in ovarian carcinoma could express CCL22 to increase Treg infiltration [27]. TAMs also release high level of IL-10 and low level of IL-12, which suppress the differentiation of Th1 cells in favor of Treg cells [28,29]. Hence, it should be examined whether tumor could promote the recruitment and differentiation of Tfr cells via similar mechanisms. Another possibility is that the ovarian carcinomas contained tertiary lymphoid structures, and the Tfr cells were attracted to these structures via CXCL13 [30].

Of note, the transcript levels of TGFB1 and IL10 in tumor-infiltrating Tfr cells were significantly higher than the transcript levels of those genes in circulating Tfr cells, suggesting that the tumor-infiltrating Tfr cells were more activated. The reason for this observed activation is unclear. Whether Tfr cells are specific to any tumor-associated antigens, or were subjected to bystander activation in the tumor microenvironment, should be examined in future experiments.

Conflict of interest

The authors declare no conflict of interest.

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