The characteristics and novel clinical implications of CD4+CXCR5+Foxp3+ follicular regulatory T cells in breast cancer

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Background: Follicular regulatory T cells (Tfr) are a subset of regulatory T cells (Tregs) that suppress the humoral immune response in the germinal center. They are associated with increased rates of disease stabilization and decreased autoantibody levels in a variety of tumor and autoimmune diseases. The binding of T-cell immunoglobulin mucin 3 (TIM-3) and its ligand on the surface of Tfr cells could result in the depletion of T lymphocytes and the termination of the immune response mediated by helper T cell 1. However, the role of Tfr cells in breast cancer (BC) remains unclear.

Methods: In this study, we detected the expression of CD4+CXCR5+Foxp3+Tfr cells in the peripheral blood of 35 BC patients and 30 healthy control patients by flow cytometry, and analyzed the relationship between Tfr cells and the clinical characteristics of patients. In addition, the expression of TIM-3 on the surface of Tfr cells in 6 triple-negative BC (TNBC) patients was further investigated using mass spectrometry.

Results: We found a significant increase in Tfr cells in BC patients compared to healthy control patients (23.47%±9.70% *vs*. 10.99%±4.68%; P=0.001). Notably, the increase was more significant in early stage than advanced stage TNBC patients (28.52%±10.75% *vs*. 18.69%±5.19%; P=0.006), and there was a negative correlation between Tfr cells and serum lactate dehydrogenase (LDH) in early stage TNBC patients (r=−0.585; P=0.008). Additionally, we found that the expression of Tfr cells was higher in TNBC patients than luminal BC patients (28.25%±10.11% *vs*. 18.5%±8.15%; P=0.028); however, there was no significant difference in expression in hormone receptor positive (HR+) BC and hormone receptor negative (HR−) BC (P=0.141) patients. Notably, the surface of Tfr cells of TNBC patients had higher levels of TIM-3 expression than those of healthy control patients (3.93±0.92 *vs*. 2.65±0.15, respectively; t=−3.02; P<0.05), which the mass spectrometry showed were positively correlated with the intracellular Foxp3 expression of Tfr cells (r=0.82; P=0.036).

Conclusions: Our results suggest that circulating Tfr cells and the expression of TIM-3 were significantly increased in BC patients, which were related to stage and histological type, and may be involved in the pathogenesis of BC.

Keywords: Breast cancer (BC); follicular regulatory T cell (Tfr); T-cell immunoglobulin mucin 3 (TIM-3)

Introduction

Follicular regulatory T cells (Tfr) cells are a recently identified class of regulatory T cells (Tregs) that maintain stable expression of ICOS, CXCR5, CD4 and Foxp3 (1). The main function of Tfr cells is to regulate humoral immunity in the germinal center by suppressing follicular helper T cells (Tfh) cells and B cells (2,3). A lack of Tfr cells might result in greater germinal center reactions (2,3). It has been confirmed that Tfr cells play a critical role in the development of non-small cell lung cancer (NSCLC), lymphoma, and other autoimmune diseases (4-6). Research has shown that Tfr cells are abundant in the lymph nodes of early stage diffuse large B cell lymphoma (DLBCL) patients, but reduced in advanced stage DLBCL patients; thus, Tfr cells may be strongly related to prognosis (7). It should be noted that T-cell immunoglobulin mucin 3 (TIM-3), one of the important immune suppressive receptors, could also co-express on the surface of Tfr cells with programmed death-1 (PD-1) (8). In the tumor microenvironment (TME), an obviously increased expression of TIM-3 could interact with T cells by containing the ligand, and be involved in the development of tumors (9,10).

Breast cancer (BC) is one of the most common malignant tumors in women, and approximately 25% of early-stage BC patients relapse within 5 years even with the implementation of surgery, chemotherapy, endocrine therapy, and other effective strategies, such as PD-1 immunotherapy (11,12). The phase III IMpassion130 trial (NCT02425891), which compared chemotherapy plus atezolizumab and chemotherapy, examined immunotherapy in the treatment of BC, and the Food and Drug Administration (FDA) has accelerated the approval of atezolizumab (13). Recently, more and more research has confirmed the inseparable relationship between the progression of BC and the immune system (14-16). Interestingly, the imbalance of Tfr/Tfh cells has been reported to be the potential cause of BC (17). It is thought that Tfr cells might synergistically promote the formation of interleukin-10 (IL-10)-produced B cells in breast carcinoma (17). Additionally, recent studies have also shown that the co-blocking of PD-1 could further upregulate the expression of TIM-3 on the surface of Treg in TNBC, and TIM-3 may be a targeted biomarker related to the resistance of PD-1 blockers (18). Tfr cells are likely to mediate the immune escape of BC through the TIM-3 pathway. Thus, the identification of key molecular targets of Tfr cells could provide a new basis for the optimization of immunotherapy.

Table 1 Clinical characteristics of breast cancer (BC) patients

Characteristics	Number (%)
Age	
<40 years	12 (34.29)
40–60 years	12 (34.29)
>60 years	11 (31.43)
TNM stage	
I–III	17 (48.57)
IV	18 (51.42)
Histological cell type	
HR–	19 (54.29)
HR+	16 (45.71)
Triple-negative BC (TNBC)	12 (34.29)
Luminal	12 (34.29)
HER-2+	7 (20.00)

In this study, we sought to clarify the characteristics of circulating Tfr (cTfr) cells and TIM-3 in BC patients by measuring the frequency of Tfr cells. We also examined the expression of immune cells in TNBC to gain insights into the function of cTfr cells in BC. We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/atm-21-3848).

Methods

Patients

Thirty-five female BC patients and 30 age- and sex-matched healthy control patients were identified at the Cancer Hospital of the University of Chinese Academy of Sciences/Zhejiang Cancer Hospital from March 2020 to September 2020. The patients were confirmed to have invasive breast carcinoma by surgical resection or puncture, and the pathological stage was classified according to the international tumor (T), nodes (N), and metastases (M) staging system for BC. Seventeen patients had early BC and 18 patients had advanced BC. In relation to the histological subtypes, 12 patients had TNBC, 12 patients had human epidermal growth factor receptor 2 positive (HER2+) BC, and 7 patients had luminal BC. The clinical characteristics of the BC patients are set out in *Table 1*. The lactate dehydrogenase (LDH) records of these patients were

Table 2 The expression of Tfr cells, B cells, and CD8+ T cells in the peripheral blood of triple-negative breast cancer (TNBC) and healthy control patients

Group	n	Tfr cells		B cells		CD8+ T cells	
		TIM-3+	Foxp3+	CD19+	IgG	CD8+	Percentage
Control	3	0.48±0.19	0.15±0.05	0.27±0.11	0.78±1.0	0.17±0.16	5.48±2.12
TNBC	6	0.96±0.40	0.20±0.09	0.12±0.10	0.28±0.17	0.05±0.03	4.42±1.94

retrospectively collected.

Additionally, 6 female TNBC and 3 age- and sex-matched healthy control patients were identified at our hospital from October 2020 to December 2020. Of these 6 patients, 3 were preoperative patients and 3 were recurrent and progressive patients.

All patients and healthy control patients were aged from 40 to 60 years old. Patients who had already received glucocorticoids, immunotherapy, or who had been diagnosed with other autoimmune diseases were excluded. 4 mL-peripheral blood samples of patients were used in this research. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional ethics board of Cancer Hospital of the University of Chinese Academy of Sciences/Zhejiang Cancer Hospital (No.: IRB-2020-14). Individual consent for this retrospective analysis was waived.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from the 4 mL-peripheral blood samples using Ficoll separation. The PBMCs were adjusted to 1×10^9/L in Roswell Park Memorial Institute Medium (RPMI) 1640 (containing 10% fetal bovine serum) and incubated with fluorophore tagged anti-human CD3, CD4, and CXCR5 for 30 min at room temperature. The cells were then washed twice with phosphate buffered saline (PBS), and intracellular Foxp3 staining was performed with the Transcription Factor Staining Buffer Kit for 50 min at 4 ℃. After fixation and permeability, anti-Foxp3 monoclonal antibody and fixed cells were incubated at 4 ℃ in the dark for 30 min. Finally, the sample were washed again and analyzed at FACS Canto II.

To determine the expression of TIM-3 on the surface of Tfr cells in TNBC, the cells were stained with anti-CD4, CXCR5, CD25, CD19, CD8, and TIM-3 for 30 mins at room temperature (see *Table 2*). The other steps were the same as those outlined above. Finally, samples were analyzed on Helios Cytof System (Fluidigm, USA). All of the antibodies used were purchased from BD Biosciences (San Diego, CA, USA).

Statistical analysis

The data are expressed as mean ± standard deviation (SD) and Boxplot. R Studio (version 3.6.2) was used for graphics production. The differences in the peripheral blood Tfr cells among different groups, BC stages, molecular types, and the expression of TIM-3 in TNBC were evaluated using *T*-tests. A P<0.05 on both sides was considered statistically significant. Flowjo was used to draw the t-distributed stochastic neighbor embedding (t-SNE) diagram to show the expression distribution and content of surface antigens in the human peripheral blood immune cells.

Results

Increased levels of Tfr cells in the peripheral blood of BC patients

To study the potential role of Tfr cells in BC, we quantitatively analyzed the differences of CD4+CXCR5+CD25+Foxp3+Tfr cells in the peripheral blood of 35 patients and 30 healthy age- and sex-matched control patients by flow cytometry (see *Figure 1A*). The patients' demographic and clinicopathologic characteristics are listed in *Table 1*. As *Figure 1B* shows, the percentage of circulating CD4+CXCR5+Foxp3+Tfr cells was significantly higher in BC patients than healthy control patients (23.47%±9.70% *vs.* 10.99%±4.68%; t=−6.59; P=0.001). We also analyzed the percentage of cTfr cells in the peripheral blood of patients with different stages of BC. Our results showed that early-stage disease BC patients had a higher percentage of cTfr cells than advanced stage BC patients (28.52%±10.75% *vs.* 18.69%±5.19%; t=3.31; P=0.006; see *Figure 1C*).
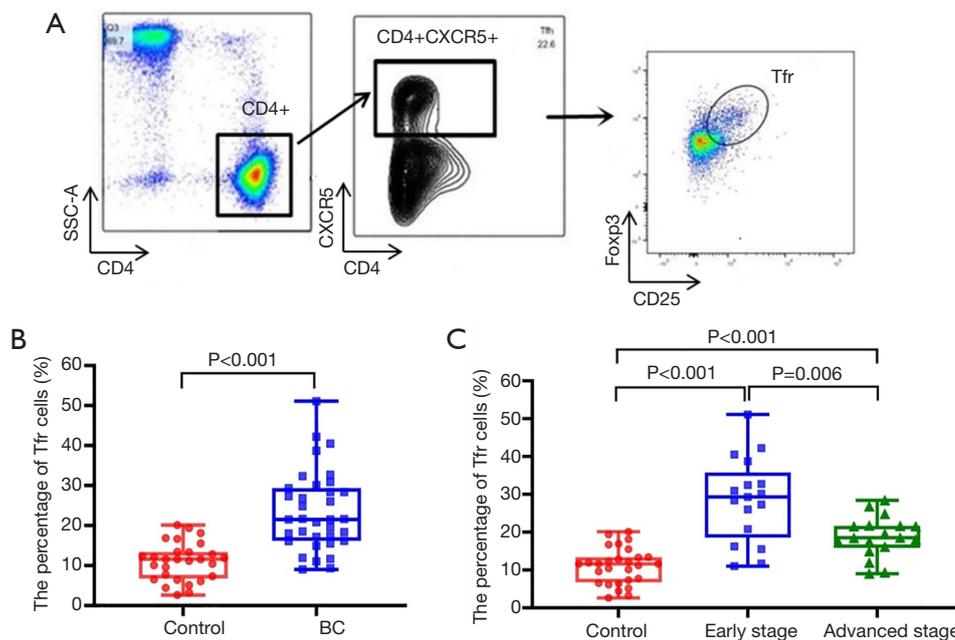
Figure 1 Circulating follicular regulatory T (cTfr) cells in breast cancer (BC) patients in different clinical stages and control patients. (A) FACS plots of CXCR5 and Foxp3 expression in CD4+ T cells from lymphocytes; (B) the percentage of cTfr cells from CD4+ T cells in BC patients and healthy control patients; (C) the percentage of cTfr cells from CD4+ T cells in early stage disease BC patients, advanced stage disease BC patients, and healthy control patients.

The relationship of cTfr cells with different histological types

We next examined the relationships between cTfr cells and molecular types in BC patients. As *Figure 2A* shows, there was no significant difference in cTfr cells between the hormone receptor positive (HR+) BC patients and hormone receptor negative (HR–) patients (26.07%±10.07% and 20.66%±8.72%, respectively; P=0.141). However, the percentages of cTfr cells in TNBC patients were significantly higher than those of HER2+and luminal BC patients (28.25%±10.11% *vs.* 22.32%±8.82%; P=0.128; 28.25%±10.11% *vs.* 18.50%±8.15%; P=0.028); however, there was no significant difference between the HER2+ and luminal BC patients (22.32 %±8.82% *vs.* 18.5%±8.15%; t=0.473; P=0.432; see *Figure 2B*).

CXCR5+Foxp3+Tfr cells in the peripheral blood of early-stage BC patients were negatively correlated with LDH

To investigate the significance of the increased percentage of cTfr cells in BC patients, we analyzed the correlation between the percentage of cTfr cells and serum LDH levels in BC patients. The results showed that the serum LDH levels of early-stage BC patients were significantly lower

than those of advanced stage BC patients (271.53±48.87 *vs.* 257.45±13.80; P=0.036). The percentage of circulating CXCR5+Foxp3+Tfr cells was significantly negatively correlated with serum LDH levels in early-stage patients (r=−0.585; P=0.008; see *Figure 3A*), but was not significantly correlated with serum LDH levels in advanced stage BC patients (P=0.875; see *Figure 3B*).

The increased expression of TIM-3 on the surface of Tfr cells in TNBC patients

To explore the significance of increased Tfr cells and the specific pathway of action in BC patients, we analyzed the expression of TIM-3 on the surface of cTfr cells in the peripheral blood of TNBC patients and healthy control patients using mass spectrometry. As *Figure 4A-4B* show, we analysed the expression of TIM-3 and Foxp3 of cTfr cells in TNBC and healthy control patients, the results revealed that the frequency of TIM-3 of BC patients was significantly higher than that of healthy patients (3.93±0.92 *vs.* 2.65±0.15, respectively; t=−3.02; P<0.05). However, there was no significant difference in the expression of TIM-3 between early-stage BC patients who had not undergone surgery
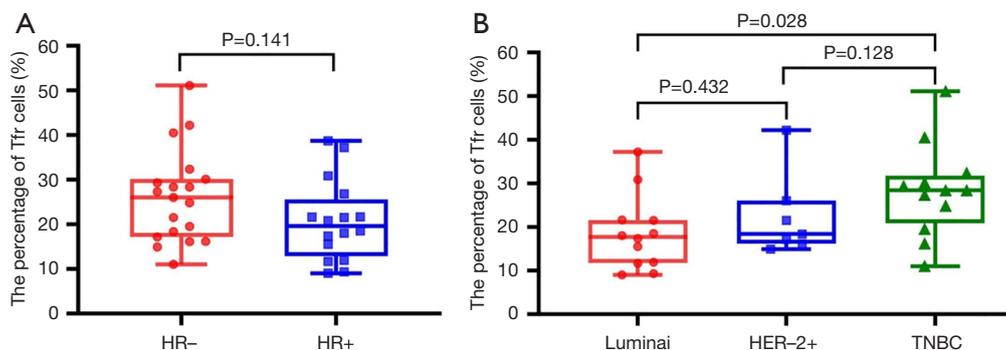
Figure 2 Circulating follicular regulatory T (cTfr) cells in breast cancer (BC) patients with histological subtype. (A) The percentage of cTfr cells from CD4+ T cells in BC patients with HR+ and HR–; (B) the percentage of cTfr cells from CD4+ T cells in triple-negative BC (TNBC), luminal BC, and HER2+ BC patients.
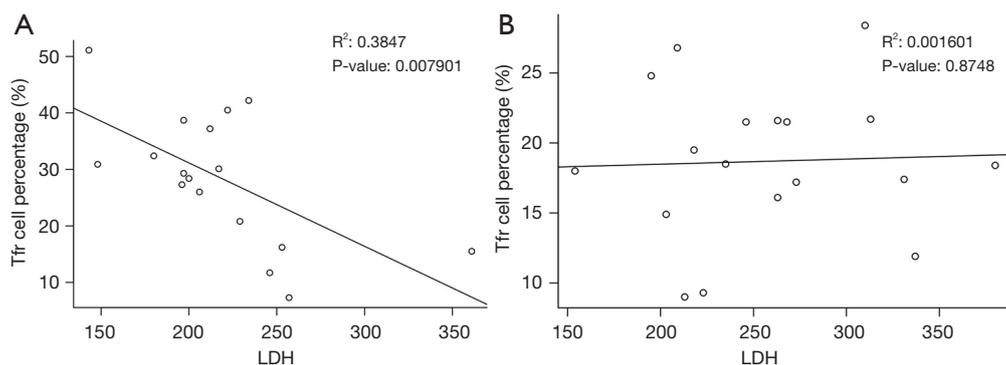

Figure 3 Correlation analysis between CXCR5+FoxP3+Tfr cells and lactate dehydrogenase (LDH) in breast cancer (BC) patients. (A) LDH levels in serum were negatively correlated with CXCR5+Foxp3+Tfr cells in early-stage BC patients; (B) the correlations were not significant between CXCR5+Foxp3+Tfr cells and LDH levels in the serum of advanced stage BC patients. Tfr, follicular regulatory T cells.

and advanced stage BC patients whose condition was evaluated as disease progression (PD). Next, we conducted a correlation analysis to examine the expression of TIM-3 and Foxp3 in TNBC patients. The results showed that there was a significant positive correlation between the expression of TIM-3 and Foxp3 in the cells (r=0.82; P=0.036; see *Figure 4C*). Furthermore, the expression level of TIM-3 in different cell clusters were shown by a Spade tree diagram (*Figure 4D*) and UMAP scatter plot (*Figure 4E*).

The expression of TIM-3 of Tfr cells in TNBC was negatively correlated with the IgG and CD19

We also conducted a correlation analysis of the expression of TIM-3 in the Tfr cells and immunoglobulin (IgG) in the serum of TNBC patients. As *Table 2* shows, the healthy control patients possessed higher levels of IgG than the TNBC patients (0.78±1.0 *vs.* 0.28±0.17; P<0.05), and the

expression of IgG was negatively correlated with TIM-3 (r=–0.98; P<0.05). We then compared the expression of CD19 on the surface of B cells in the peripheral blood between TNBC and healthy control patients. Similar to IgG, the expression of CD19 of TNBC patients was significantly lower than that of healthy control patients (0.12±0.10 *vs.* 0.27±0.11; P<0.05), and it was negatively correlated with TIM-3 expression on the surface of Tfr cells (r=–0.98; P<0.05). However, in relation to the analysis of CD8, no significant correlations were found.

Discussion

In this study, we found that the level of CXCR5+FoxP3+Tfr cells was increased in the peripheral blood of BC patients, especially in early-stage BC and TNBC patients. Further, we found a positive correlation between CXCR5+FoxP3+Tfr cells and LDH. Additionally, we
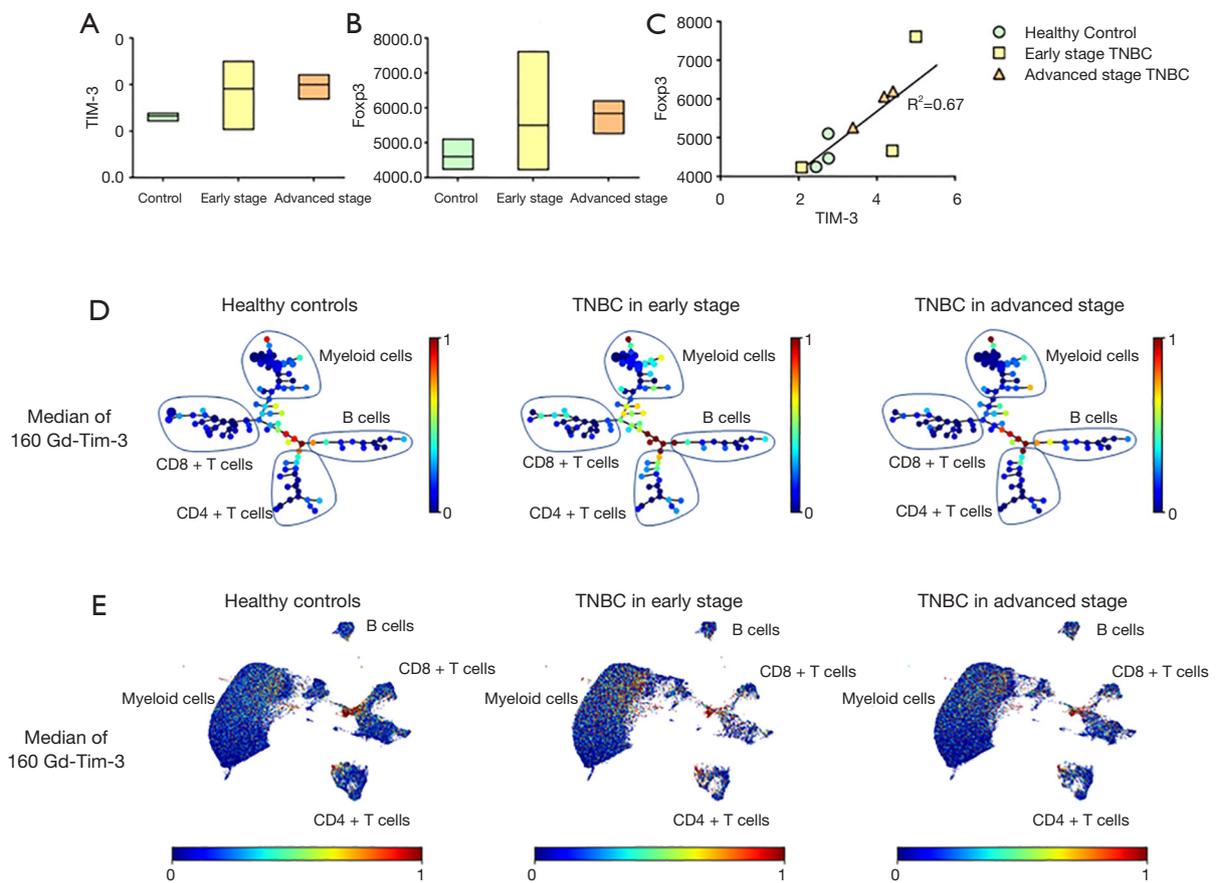
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Figure 4 The expression of T-cell immunoglobulin mucin 3 (TIM-3) on the surface of circulating follicular regulatory T (cTfr) cells in triple-negative breast cancer (TNBC). (A) The expression of TIM-3 on the surface of cTfr cells in TNBC and healthy control patients; (B) the expression of Foxp3 of cTfr cells in TNBC and healthy control patients; (C) the correlation analysis between TIM-3 expression and Foxp3; (D,E) the expression level of TIM-3 in different cell clusters were shown by a Spade tree diagram (D) and UMAP scatter plot (E).

showed that the expression level of TIM-3 on the surface of Tfr cells was also significantly higher in TNBC patients than healthy control patients, and was negatively correlated with the related auto-antibodies. Our results provide significant insights into the role of Tfr cells in the pathogenesis process of BC.

Studies have shown that Tfr cells play an important role in the development of autoimmune diseases and tumor diseases; for example, the frequency of FoxP3+CXCR5+CD4+Tfr cells in the peripheral blood of diffuse large B cell lymphomas(DLBCL) patients was reported to be significantly increased compared to that of healthy control patients, especially in patients with less advanced stages of DLBCL, and the Tfr cells may be involved in intratumoral immunity (6). Additionally, the results of research on Tfr cells in NSCLC are similar to those for DLBCL. However, very few studies have been conducted on the function of

Tfr cells in BC. Indeed, to date, only Hongsheng Song *et al.* appeared to have examined the expression and characteristics of Tfr cells in BC (17). In the present study, we found an increase of cTfr cells in BC patients, and that the number of Tfr cells was significantly higher in early-stage BC patients than advanced stage. Thus, elevated cTfr cells might be a response to the pathogenesis of BC, and might be related to the disease stage, such that patients with early-stage BC may have stronger immunosuppression than advanced stage BC patients.

We also retrospectively collected the serum LDH records of these patients, and found that LDH levels in early-stage BC patients were significantly lower than LDH levels in advanced stage BC patients. However, we also found that higher CXCR5+FoxP3+Tfr levels were correlated with lower levels of LDH in early-stage BC patients. LDH is an enzyme that catalyzes the interconversion of pyruvate and

lactate, which in turn promotes cancer cell invasion, anoikis resistance, and tumor metastasis (19). Several studies have reported that LDH is a poor prognostic factor in BC (20,21). Further, it has been suggested that LDH depletion inhibits the formation of metastases and prolongs the survival of mice with breast tumors by regulating the TME and modulating the immune response (20,21). Tfr cells may be a better indicator of immune suppression in the germinal center of early BC patients than LDH. Notably, there were no significant correlations between CXCR5+FoxP3+cells and LDH levels in advanced stage BC patients; however, these results could be related to this study's small sample size and the different metastatic sites.

Compared to luminal BC, TNBC and HER2+ BC have higher tumor infiltrating lymphocytes (TILs) (22,23). TIL infiltration is closely related to the prognosis of BC patients and their response after immunotherapy. Numerous studies have shown that the expression of immune checkpoints in tumor cells with high TIL infiltration may be higher that of tumor cells with low TIL infiltration (14,24). In addition, according to a report of Saleh *et al.* (18), the co-blockade of PD-1 and programmed death-ligand 1 (PD-L1) could further upregulate the co-expression of TIM-3 and Lymphocyte activation gene-3 (LAG-3) on CD4+CD25+ T cells and CD4+CD25+Foxp3+Tregs in the presence of TNBC cells. Our results showed that CXCR5+FoxP3+Tfr cells were significantly more abundant in TNBC patients than HER2+ and luminal BC patients; however, no significant difference was found between TNBC and HER2+ BC patients.

Most importantly, we showed that the expression of TIM-3 on the surface of Tfr cells was significantly higher than that of healthy control patients. We also observed a positive relationship between the expression of TIM-3 and Foxp3, which distinguishes Tfr cells from Tfh cells, and a negative correlation between the expression of TIM-3 and IgG on the surface of B lymphocytes. Thus, we speculate that the elevated expression of TIM-3 on the surface of Tfr cells might mediate tumor immune suppression in BC patients through the surface TIM-3, and affect the proliferation of effector cells in the germinal center, thus inhibiting the occurrence of humoral immunity.

Overall, our study showed an increased percentage of CXCR5+FoxP3+Tfr cells in BC patients. We theorize that this might play an important function in the pathogenesis of BC. Our research on the pathway of action of Tfr cells in 6 TNBC patients was only preliminary; thus, the potential mechanisms require further research.

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://dx.doi.org/10.21037/atm-21-3848

Data Sharing Statement: Available at https://dx.doi.org/10.21037/atm-21-3848

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/atm-21-3848). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional ethics board of Cancer Hospital of the University of Chinese Academy of Sciences/Zhejiang Cancer Hospital (No.: IRB-2020-14). Individual consent for this retrospective analysis was waived.

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