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ORIGINAL ARTICLE

T follicular regulatory cells suppress Tfh-mediated B cell help and synergistically increase IL-10-producing B cells in breast carcinoma

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Abstract

T follicular regulatory (Tfr) cell is a recently discovered subset of T regulatory (Treg) cells. The main function of Tfr cells is thought to suppress germinal cancer reaction and inhibit B cell proliferation and Ig production. However, recent studies demonstrate that Tfr cells may be required for high-affinity Ig formation during acute virus infections. The role of Tfr cells in breast cancer is not thoroughly investigated. In this study, total circulating CD4 T cells were sorted into $CD25^{+}CXCR5^{-}$ Treg-like, $CD25^{+}CXCR5^{+}$ Tfr-like, and $CD25^{-}CXCR5^{+}$ Tfh-like subsets. Data showed that the Tfr-like subset presented intermediate levels of both Foxp3 and Bcl-6, while the Treg-like subset was high in Foxp3 and low in Bcl-6, and the Tfh-like was high in Bcl-6 and low in Foxp3. Of note, the frequencies of Tfr-like and Treg-like cells were significantly elevated in breast cancer (BC) patients than in non-cancer (NC) controls. Tfr-like cells in BC patients also expressed significantly higher levels of Foxp3 than those in NC controls. Neither Treg-like nor Tfr-like cells could support Ig production from naive B cells, while Tfh-like cells potently supported Ig production from naive B cells. Tfr-like cells increased the availability of IL-10, both by directly producing IL-10 and by increasing IL-10 production from B cells. Interestingly, Tfr-like cells increased IL-10 production from B cells synergistically with Tfh cells, but at the same time, significantly reduced Ig production in the Tfh-B cell coculture. These Tfr-mediated effects on Tfh cells were not found in canonical Treg cells. Overall, this study demonstrates several distinctive features in circulating Tfr cells and suggests that Tfr cells may promote the formation of IL-10-producing B cells in BC.

Keywords B cell · Breast cancer · IL-10 · Tfr cell

Introduction

The human canonical T regulatory (Treg) cells are identified by $CD3^{+}CD4^{+}CD25^{high}Foxp3^{+}$ expression and can be found in the peripheral blood, secondary lymphoid organs, and various epithelial tissues, such as the lung, skin, and the gastrointestinal tract [1]. By expressing CD25 (IL-2 receptor α subunit), Treg cells can act as an IL-2 sink, reducing the availability of IL-2 to activated T effector (Teff) cells [2]. Treg cells also express CTLA-4, which suppresses costimulatory signals from CD80 and CD86 [3, 4]. Additionally, Treg cells may release regulatory cytokines, such as IL-10 and TGF-β, to dampen inflammation and express cytotoxic molecules, such as granzyme and perforin, to eliminate Teff cells and natural killer (NK) cells [4, 5]. These Treg-mediated mechanisms are critical for tissue protection and peripheral tolerance but can also play a deleterious role by suppressing Teff responses during virus infections and cancer [6].

In recent years, a Treg subtype with surface CXCR5 expression is discovered and has been termed T follicular regulatory (Tfr) cells [7, 8]. Tfr cells express Bcl6 to a level that is lower than that in T follicular helper (Tfh) cells but higher than that in other T cell subsets [9]. Tfr cells also express the Bcl-6 antagonist Blimp-1, which is not expressed by Tfh cells [10]. It is thought that the main function of Tfr cells is to suppress

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Tfh cell and B cell proliferation during germinal center reactions and to promote the generation of high-affinity antibodies [7, 11–13]. In a model where the Bcl-6 gene is deleted in Foxp3$^+$ Treg cells, the mice presented enhanced protection against the influenza virus, as well as elevated disease markers of experimental Sjögren's syndrome, an autoimmune condition characterized by high production of autoantibodies [14]. However, another study using the same model demonstrated that Tfr cells promoted germinal center responses during acute viral infection via the provision of IL-10 [15], suggesting that the functions of Tfr cells are multifaceted and context-dependent. In blood, circulating Tfr-like cells have been discovered, and the reduction in circulating Tfr to Tfh ratio is associated with the development of autoimmune disease [16–18].

Several studies have investigated Tfr cells in malignancies. In human follicular lymphoma, it was shown that the mesenchymal stromal cells in the tumor microenvironment were capable of inducing Tfr cells by upregulating Foxp3 expression in Tfh cells [19, 20]. In addition, Tfr cells were enriched in malignant lymph nodes. In diffuse large B cell lymphoma, Tfr cells were found at varying frequencies in the enlarged lymph nodes and were capable of suppressing CD4$^+$CD25$^-$ T cell and CD8 T cell proliferation [21]. In ovarian cancer patients, Tfr cells infiltrated the tumor, expressed regulatory molecules, and suppressed CD8 T cell activation in vitro [22].

In this study, we investigated the characteristics of Tfr cells in breast cancer (BC) patients. First, circulating Tfr cells were identified by the expression of surface markers and transcription factors. The functional characteristics of Tfr cells were then compared between BC subjects and non-cancer control (NC) subjects. Finally, the effect of Tfr cells on Tfh-mediated B cell help was examined.

Methods

Participants

This study was approved by the ethics committee of the Second People's Hospital of Dongying and was performed in accordance with the Declaration of Helsinki. All BC patients were newly diagnosed at the Second People's Hospital of Dongying. Peripheral blood was obtained from all individuals once the written informed consent was received. All patients were untreated prior to sample collection. Controls were composed of 30 age-matched females, who had no medical history of breast cancer or any other forms of malignancy. The exclusion criteria, which applied to both the BC group and the NC group, were age outside of the 18–65 range, autoimmunity, inflammatory bowel disease, acute or chronic infections, and/or presenting terminal diseases.

Flow cytometry

Fresh peripheral blood mononuclear cells (PBMCs) were isolated from blood using Ficoll-Paque PLUS (GE Healthcare) gradient centrifugation. PBMCs were then incubated with LIVE/DEAD Fixable Violet Stain (Invitrogen) for 30 min, followed with anti-human CD3, CD4, CXCR5, and CD25 monoclonal antibodies (BioLegend) for 30 min. Subsequently, PBMCs were washed and incubated with FOXP3 FIX/PERM Buffer (BioLegend) for 20 min, and then incubated with anti-human Foxp3 and anti-human Bcl-6 (BioLegend) for 30 min. The PBMCs were washed again and were acquired using the FACS Canto instrument (BD Biosciences). Data analysis was performed in FlowJo (Tree Star).

Cell isolation and sorting

Total CD4 T cells were sorted using Human CD4 T Cell Enrichment Kit (Stemcell Technologies), with purities above 97.5% (confirmed using anti-human CD3-positive and CD4-positive staining). Purified CD4 T cells were then stained with anti-human CD25 and CXCR5 for 30 min, washed, and sorted into CD25$^+$CXCR5$^-$, CD25$^+$CXCR5$^+$, and CD25$^-$CXCR5$^+$ subsets in the FACS Aria instrument (BD Biosciences). Naive B cells were sorted using Human Naive B cell Enrichment Kit (Stemcell Technologies), with purities above 97% (confirmed using anti-human CD19-positive and CD27-negative staining).

T cell-B cell coculture

In a 96-well round-bottom plate (Corning), naive B cells and sorted CD4 T cell subsets were incubated at 2×10^4 cells per well per cell type. A total of 1 μg/mL endotoxin-reduced SEB (Sigma) was added to RPMI 1640 complete medium, which was then added to the cells at 200 μL per well. After 12 days, the supernatant was separated from the cells via centrifugation. The cell pellet was then resuspended in sterile phosphate-buffered saline supplemented with 2% heat-inactivated fetal bovine serum, and divided into two parts. T cells and B cells were isolated individually from each part using Human CD4 T Cell Enrichment Kit and Human B cell Enrichment Kit (BioLegend), respectively.

Ig and IL-10 measurement

The concentrations of IgM, IgG, and IL-10 in the supernatant were measured using the Human IgM ELISA Kit, the Human IgG ELISA Kit (both from Abcam), and the Human IL-10 Quantikine ELISA Kit (R&D Systems), respectively.

IL-10 ELISpot

Live sorted T cells and B cells from the T-B coculture were first counted in the presence of Trypan Blue Dye (Invitrogen), and then incubated at various dilutions in a PVDF plate precoated with IL-10 capture antibody from the Human IL-10 ELISpot Kit (R&D Systems). The rest of the experiments followed instructions from the kit. The frequency of IL-10-producing cells was calculated as the number of positive spots divided by the number of live cells multiplied by 100%.

Statistics

Data were represented as scatterplots and/or mean ± SEM. Figure making and statistical tests were performed using Prism (GraphPad). All tests were two-tailed, and $p < 0.05$ was required for significance. Specific tests applied were listed in the figure legends or in the text.

Results

Tfr frequency in peripheral blood

In peripheral blood $CD4^+$ T cells, we examined the expression of CD25, a marker of canonical Treg cells, and of CXCR5, a marker of circulating Tfh-like cells [23], using flow cytometry staining $CD25^+CXCR5^-$, $CD25^+CXCR5^+$, and $CD25^-CXCR5^+$ cells in total $CD3^+CD4^+$ T cells were gated as shown (Fig. 1a). The frequencies of $CD25^+CXCR5^-$ CD4 T cells were at 6.3% ± 0.5% (mean ± SEM hereafter) in NC subjects and slightly higher at 8.7% ± 0.7% in BC subjects (Fig. 1b, left panel). The frequencies of $CD25^+CXCR5^+$ CD4 T cells were at 1.2% ± 0.2% in NC subjects and markedly higher at 3.7% ± 0.4% in BC subjects (Fig. 1b, middle panel). The frequencies of $CD25^-CXCR5^+$ CD4 T cells were at 8.7% ± 0.3% in NC subjects and at 9.7% ± 0.5% in BC subjects, with no significant difference between the two groups (Fig. 1b, right panel).

To verify the identity of the $CD25^+CXCR5^-$, $CD25^+CXCR5^+$, and $CD25^-CXCR5^+$ CD4 T cells, the expression of Treg transcription factor Foxp3 and Tfh transcription factor Bcl-6 was examined (Fig. 2a, c). In both NC subjects and BC subjects, the expression of Foxp3 was highest in $CD25^+CXCR5^-$ cells, intermediate in $CD25^+CXCR5^+$ cells, and lowest in $CD25^-CXCR5^+$ cells ($p < 0.01$ for all pair-wise comparisons; Fig. 2b). Interestingly, the Foxp3 expression in $CD25^+CXCR5^-$ cells and in $CD25^-CXCR5^+$ cells was not significantly different between NC subjects and BC subjects, but the Foxp3 expression in $CD25^+CXCR5^+$ cells was significantly higher in BC subjects than in NC subjects (Fig. 2b). The expression of Bcl-6, on the other hand, was the lowest in $CD25^+CXCR5^-$ cells, intermediate in $CD25^+CXCR5^+$ cells, and the highest in $CD25^-CXCR5^+$ cells ($p < 0.05$ for all pair-wise comparisons; Fig. 2d). Additionally, the expression of Bcl-6 in NC $CD25^-CXCR5^+$ cells was significantly higher than that in BC $CD25^-CXCR5^+$ cells (Fig. 2d).

Overall, these data demonstrated that the transcription factor expression by circulating $CD25^+CXCR5^-$, $CD25^+CXCR5^+$, and $CD25^-CXCR5^+$ CD4 T cells was consistent with that in the canonical Treg cells and germinal center Tfr and Tfh cells, respectively [8]. In addition, circulating Tfr cells and Treg cells were significantly elevated in BC subjects than in NC subjects.

Tfr cell functional characteristics

Both germinal center Tfh cells and circulating Tfh-like cells could support B cell-mediated Ig expression in vitro [24]. The germinal center Tfr cells, on the other hand, were shown to suppress Ig production and Tfh cell-mediated B cell help. The function of circulating Tfr-like cells is less clear. BC subjects presented elevated levels of circulating Tfr cells, granting availability for functional studies. First, we investigated the function of circulating Tfr cells on Ig production from B cells. In 20 BC patients, whose frequency of Tfr cells was greater than 3% in total CD4 T cells, we sorted circulating Treg, Tfr, and Tfh cells as $CD25^+CXCR5^-$, $CD25^+CXCR5^+$, and $CD25^-CXCR5^+$ CD4 T cells, respectively, as shown in Fig. 1a. Each subset was then coincubated with autologous naive B cells in the presence of superantigen SEB, for 12 days. Subsequently, the supernatant was separated using centrifugation, and the B cells were isolated via negative selection. The secretion of Ig was evaluated in the supernatant using Ig ELISA, while the proliferation of B cells was measured using thymidine incorporation. The expression of IgM and IgG was significantly higher in the Tfh-B cell coculture than that in the Treg-B cell coculture and the Tfr-B cell coculture (Fig. 3a). Also, the B cell proliferation potential was significantly higher in the Tfh-B cell coculture than that in the Treg-B cell coculture and the Tfr-B cell coculture (Fig. 3b). No significant differences between the Treg-B cell coculture and the Tfr-B cell coculture were observed (Fig. 3a, b).

Subsequently, we performed a separate Tfh-B cell coculture experiment, and in some experiments, autologous Tfr cells or Treg cells were added in addition to the Tfh cells at a 1-to-1 ratio. Stimulation, supernatant and B cell collection, and ELISA and thymidine incorporation assays were then performed as previously described. Interestingly, IgM and IgG concentrations were significantly reduced if the Tfr cells, but not the Treg cells, were added to the Tfh-B cell coculture (Fig. 3c). Addition of Tfr or Treg cells to the Tfh-B cell coculture did not affect the proliferation of B cells (Fig. 3d).

Overall, these data demonstrated that Tfh, but not Tfr or Treg cells, could promote the production of Ig and the proliferation of B cells. Tfr cells were capable of suppressing Ig expression in Tfh-B cell cocultures but were unable to suppress B cell proliferation in Tfh-B cell cocultures.

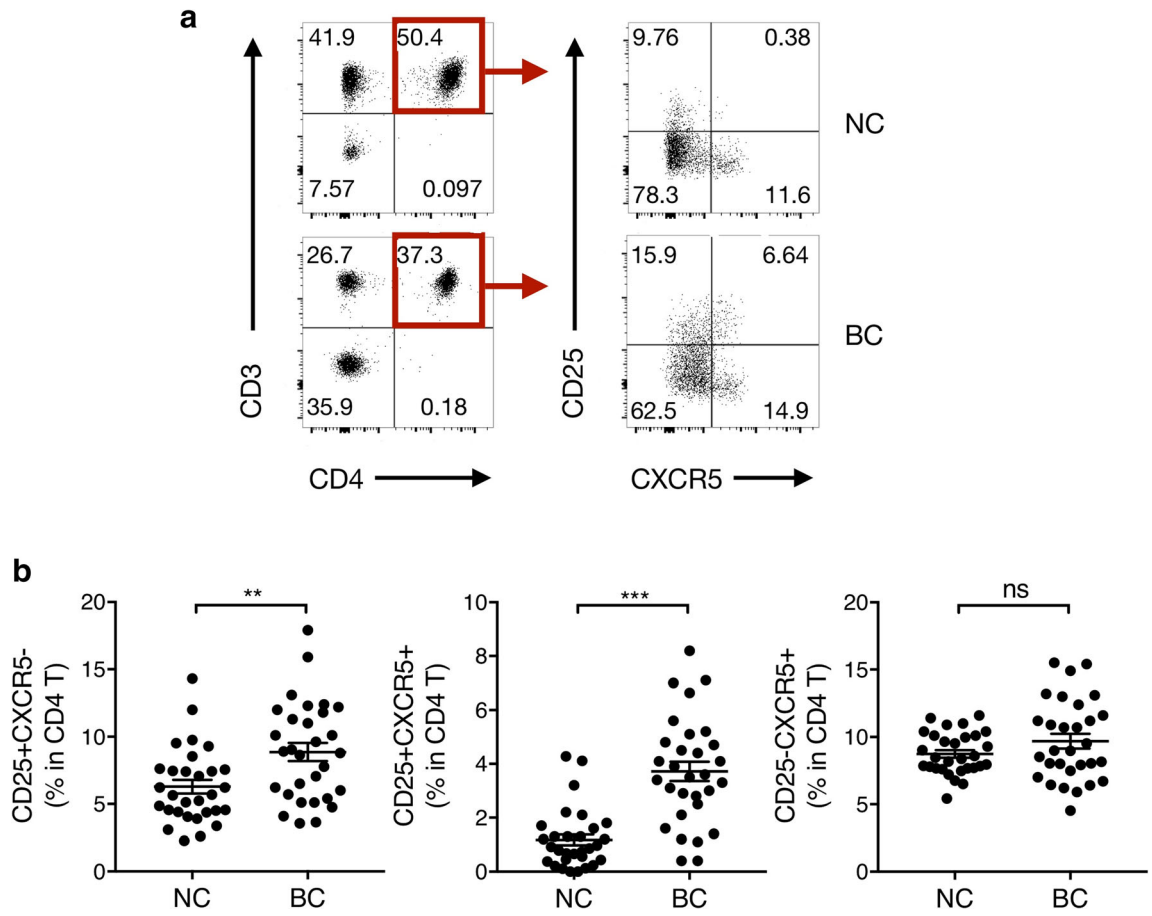

Fig. 1 Expression of CD25 vs. CXCR5 in circulating CD4 T cells. **a** Gating of CD4 T cells (left panels) and the expression of CD25 vs. CXCR5 in CD4 T cells (right panels). Figures shown were from one representative NC subject and one representative BC subject. **b** The frequencies of $CD25^{+}CXCR5^{-}$ (left panel), $CD25^{+}CXCR5^{+}$ (middle panel), and $CD25^{-}CXCR5^{+}$ (right panel) cells as a percentage of total CD4 T cells. Data were collected from 30 NC subjects and 30 BC subjects. Student's *t* test. Ns, not significant. $^{**}p < 0.01$; $^{***}p < 0.001$

Tfr cells increased IL-10 availability

In the supernatants from the Treg-B, Tfr-B, and Tfh-B cell cocultures (Fig. 4a, left panel) and the Tfh-B, Tfr+Tfh-B, and Treg+Tfh-B cocultures (Fig. 4a right panel), we examined IL-10 concentration. Interestingly, the Tfr-B coculture supernatant contained significantly higher levels of IL-10 than the Treg-B and the Tfh-B coculture supernatants (Fig. 4, left panel), and the Tfr+Tfh-B coculture supernatant contained significantly higher levels IL-10 than the Tfh-B and the Treg+Tfh-B coculture supernatant (Fig. 4, right panel), indicating that the presence of Tfr cells resulted in higher IL-10 concentration.

Tfr cells increased IL-10 production from B cells synergistically with Tfh cells

To determine the source of IL-10, we repeated the T-B coculture experiments as described above, with the modification that at the end of the coculture, CD4 T cells and B cells were separated via magnetic negative selection. The frequencies of IL-10-producing CD4 T cells and IL-10-producing B cells were evaluated using ELISpot. Tfr cells presented significantly higher IL-10 production than either Treg cells or Tfh cells, and Tfh cells presented significantly higher IL-10 production than Treg cells (Fig. 5a, left panel). Interestingly, the frequency of IL-10-producing B cells in the Tfr-B coculture was significantly higher than that in the Tfh-B coculture and that in the Treg-B coculture (Fig. 5a, right panel).

In addition, to examine whether Tfr cells affected the interaction between Tfh cells and B cells, we repeated the Tfh-B, Tfr+Tfh-B, and Treg+Tfh-B coculture experiments, and evaluated the frequencies of IL-10-producing T cells and of IL-10-producing B cells as described above. Similar to the discovery in Fig. 5a, the presence of Tfr cells significantly increased the frequencies of IL-10-producing T cells (Fig. 5b, left panel) and the frequencies of IL-10-producing B cells (Fig. 5b, right panel). Interestingly, the frequency of IL-10-producing T cells in the Tfr+Tfh-B coculture was significantly higher than that in the Tfr-B coculture ($p < 0.001$; paired *t* test), and the frequency of IL-10-producing B cells in the Tfr+Tfh-B coculture was also significantly higher than that in the Tfr-B coculture ($p < 0.001$; paired *t* test).

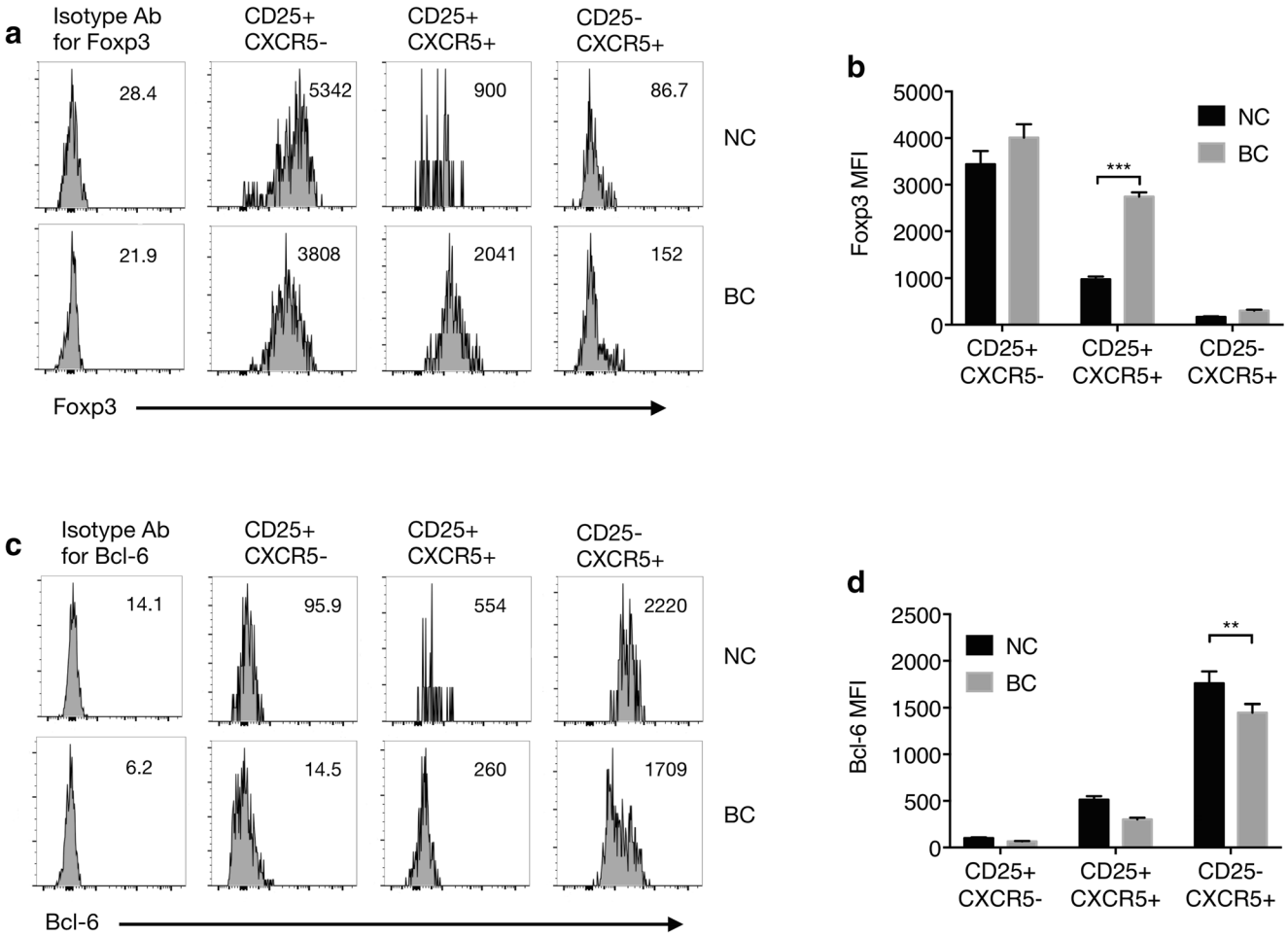

Fig. 2 Foxp3 and Bcl-6 expression in CD4 T cell subsets. **a** Foxp3 expression in $CD25^+CXCR5^-$, $CD25^+CXCR5^+$, and $CD25^-CXCR5^+$ CD4 T cells. Numbers represent the MFI of Foxp3 in each figure. Figures shown were from one representative NC subject and one representative BC subject. **b** Foxp3 MFI in $CD25^+CXCR5^-$, $CD25^+CXCR5^+$, and $CD25^-CXCR5^+$ CD4 T cells from 30 NC subjects and 30 BC subjects. Two-way ANOVA followed by Tukey's and Sidak's multiple comparisons. $^{**}p < 0.01$. **c** Bcl-6 expression in $CD25^+CXCR5^-$, $CD25^+CXCR5^+$, and $CD25^-CXCR5^+$ CD4 T cells. Numbers represent the MFI of Foxp3 in each figure. Figures shown were from one representative NC subject and one representative BC subject. **d** Bcl-6 MFI in $CD25^+CXCR5^-$, $CD25^+CXCR5^+$, and $CD25^-CXCR5^+$ CD4 T cells from 30 NC subjects and 30 BC subjects. Two-way ANOVA followed by Tukey's and Sidak's multiple comparisons. $^{**}p < 0.01$; $^{***}p < 0.001$

Discussion

Tfr cells are a recently characterized Treg subset with both Tfh-like and Treg-like features. The phenotypical and functional characteristics of Tfr cells vary slightly or considerably across different studies, in a manner that is dependent on the source organism, localization, animal model, and disease conditions. For example, in mice, it is thought that Tfr cells arose from Treg precursors but not from Tfh cells [8]. However, in human follicular lymphoma patients, mesenchymal stromal cells were capable of inducing both CD25 and Foxp3 expression in Tfh cells, effectively converting Tfh cells into Tfr cells [19]. Also, the main function of Tfr cells is thought to suppress germinal center reactions, because in mice that were adoptively transferred with Bcl6-deficient or CXCR5-deficient Treg cells (no Tfr cells), the antigen-specific B cell frequency, serum Ig concentration, plasma cell number, and germinal center B cell number were all significantly increased [7, 11]. Also, human Tfr ($CD4^+CXCR5^+CD25^+CD127^-$-sorted) cells, when added to the Tfh-memory B cell coculture, could attenuate the production of IgA [25]. However, in mice with Bcl-6-deleted $Foxp3^+$ Treg cells, IgG production was significantly decreased [26]. In addition, Tfr cells could act as a source of IL-10 during germinal center reaction. In the absence of IL-10-expressing Tfr cells, the level of lymphocytic choriomeningitis-specific Ig was significantly reduced [15].

In our study, we focused on the properties of Tfr cells in breast cancer patients. Using CD25 and CXCR5 expression, we distinguished total CD4 T cells into Treg-like $CD25^+CXCR5^-$, Tfr-like $CD25^+CXCR5^+$, and Tfh-like $CD25^-CXCR5^+$ subsets. The expression of Foxp3 and Bcl6 in each subset was consistent with canonical Treg, Tfr, and Tfh cells, thus confirming their identity. Remarkably, the Tfr-like $CD25^+CXCR5^+$ fraction was significantly elevated in BC patients than in NC subjects.

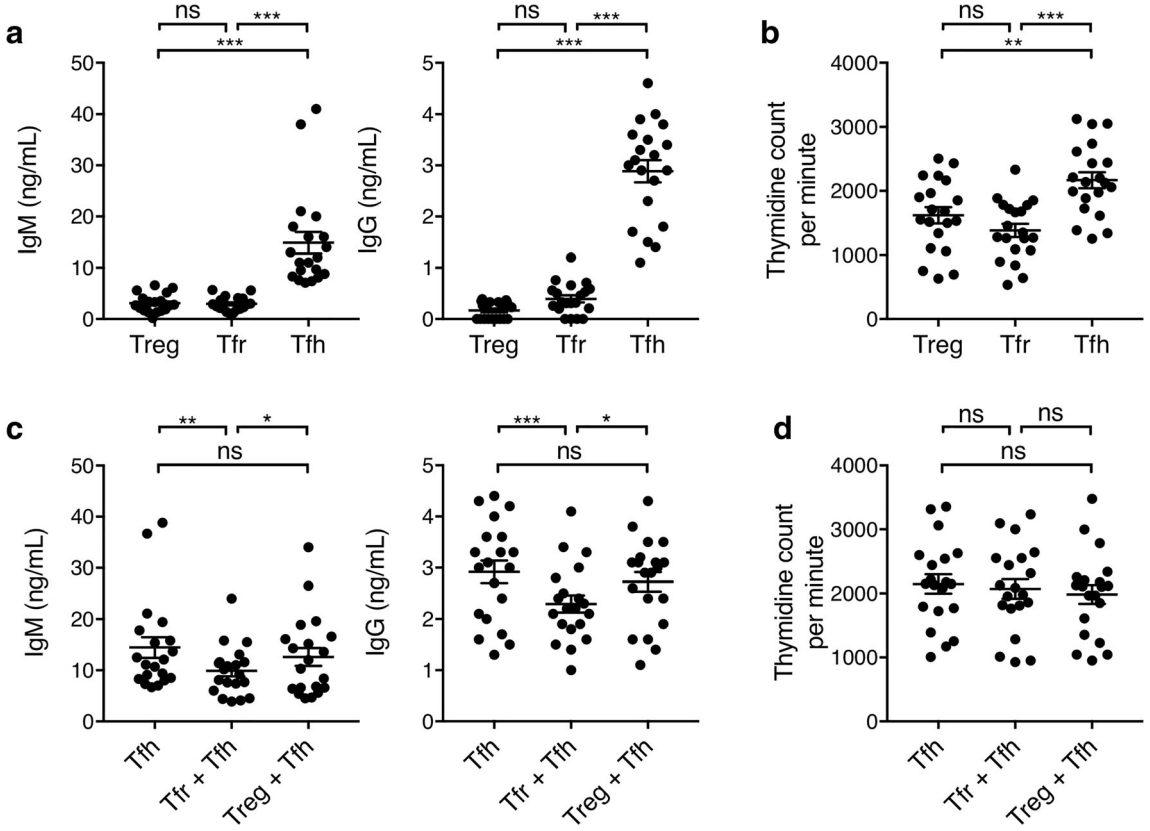

Fig. 3 Treg, Tfr, and Tfh-mediated effect on B cell inflammation. **a**, **b** Treg ($CD25^+CXCR5^-$), Tfr ($CD25^+CXCR5^+$), and Tfh ($CD25^-CXCR5^+$) CD4 T cells were incubated with naive B cells at a 1-to-1 ratio, in the presence of 1 μg/mL SEB for 12 days. The supernatant and the B cells were then collected. **a** The concentration of IgM (left panel) and IgG (right panel) in the supernatant. **b** The radioactivity of B cells after pulsing with 0.1 μCi/mL [H^3]-thymidine for 6 h. One-way ANOVA followed by Tukey's test. Ns, not significant. $^{**}p < 0.01$; $^{***}p < 0.001$. **c**, **d** Tfh cells alone, or Tfh cells in the presence of Tfr cells or Treg cells, were coincubated with naive B cells in the presence of 1 μg/mL SEB for 12 days. All cell subsets were added at a 1-to-1 ratio. The supernatant and the B cells were then collected. **c** The concentration of IgM (left panel) and IgG (right panel) in the supernatant. **d** The radioactivity of B cells after pulsing with 0.1 μCi/mL [H^3]-thymidine for 6 h. RM one-way ANOVA followed by Tukey's test. Ns, not significant. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

Additionally, the Tfr-like $CD25^+CXCR5^+$ fraction from BC patients presented higher Foxp3 than the Tfr-like $CD25^+CXCR5^+$ fraction from NC controls. Tfr cells also presented Bcl-6 at a level intermediate between Treg cells and Tfh cells.

In our in vitro system, we found that Tfr cells were unable to induce high Ig secretion alone but were able to significantly suppress Tfh-mediated help of Ig secretion, which was consistent with the other human in vitro

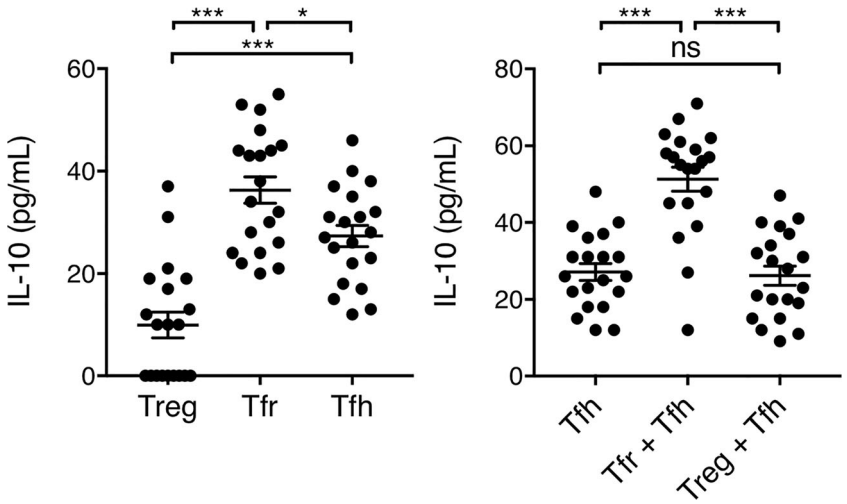

Fig. 4 IL-10 expression in T-B coculture supernatant. The concentration of IL-10 in the supernatant from Treg-B, Tfr-B, and Tfh-B cell cocultures (left panel), and from Tfh-B, Tfr+Tfh-B, and Treg+Tfh-B cocultures (right panel). One-way ANOVA followed by Tukey's test. Ns, not significant. $^{*}p < 0.05$; $^{***}p < 0.001$

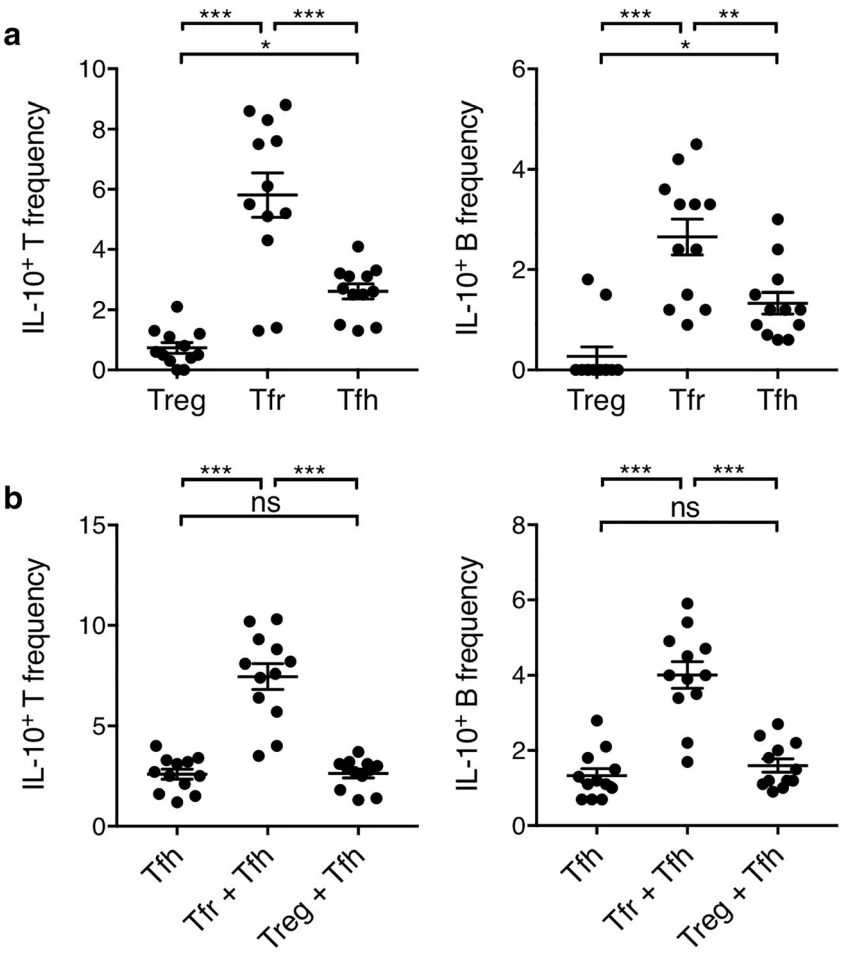

Fig. 5 IL-10 expression by T cells and B cells. **a** The Treg-B, Tfr-B, and Tfh-B cell cocultures were performed as described in Fig. 3a and b. The T cells and B cells were separated at the end of the coculture and an ELISPOT was performed to determine the frequency of IL-10^+ T cells (left panel) and the frequency of IL-10^+ B cells (right panel). One-way ANOVA followed by Tukey's test. Ns, not significant. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **b** The Tfh-B, Tfr+Tfh-B, and Treg+Tfh-B cell cocultures were performed as described in Fig. 3c and d. The T cells and B cells were separated at the end of the coculture and an ELISPOT was performed to determine the frequency of IL-10^+ T cells (left panel) and the frequency of IL-10^+ B cells (right panel). One-way ANOVA followed by Tukey's test. Ns, not significant. *$p < 0.05$; ***$p < 0.001$

discoveries [25]. Furthermore, this capacity was restricted to the Tfr fraction, but not the Treg fraction. Tfr cells had no effect on B cell proliferation alone or in combination with Tfh cells. At the same time, Tfr cells significantly elevated the availability of IL-10, both by directly producing IL-10 and by indirectly inducing IL-10 production from B cells. Importantly, a synergistic effect between Tfr cells and Tfh cells was observed, as the frequencies of IL-10-producing B cells and IL-10-producing T cells were significantly higher in the Tfr+Tfh-B cell coculture than in either the Tfr-B cell coculture or the Tfh-B cell coculture.

Further studies are required to examine the implications of these findings. For example, it has been shown that IL-10 is critical to B regulatory (Breg) cell-mediated suppression, as well as to B cell proliferation and Ig production [27, 28]. We showed that Tfr cells promoted high IL-10 production from B cells but did not result in concurrent high Ig production. Whether these B cells, after coculture with Tfr cells, derived into Breg-like cells with the potential to suppress anti-tumor immunity remains unknown. In addition, the phenotypical and functional characteristics of Tfr cells in the intratumoral environment are yet unclear and should be further explored.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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