

## Increased Frequency of Follicular Helper T Cells in Patients with Autoimmune Thyroid Disease

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**Context:** Follicular helper T (T<sub>fh</sub>) cells exert an important role in the autoimmune diseases.

**Aim:** Our study aimed to explore the role of T<sub>fh</sub> cells in patients with autoimmune thyroid disease (AITD).

**Design:** T<sub>fh</sub> cell is a new subset regulating the antibody production of B cell. Previous studies implicated CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> or CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>high</sup> as the markers of circulating T<sub>fh</sub> cells. Sixty-five patients with AITD and 30 healthy controls were enrolled in the current study. The percentages of circulating T<sub>fh</sub> cells were assessed by flow cytometry. The correlation between the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells and the levels of autoantibodies or hormones was also analyzed. Additionally, polyphasic methods were applied to investigate the status of T<sub>fh</sub> cells in thyroid glands of Hashimoto's thyroiditis patients.

**Results:** Increased percentages of circulating T<sub>fh</sub> cells in AITD patients were detected, and a positive correlation between the percentages of circulating T<sub>fh</sub> cells and the serum concentrations of anti-TSH receptor-Ab/thyroperoxidase-Ab/thyroglobulin-Ab was confirmed. A positive or modest relationship between the percentages of circulating T<sub>fh</sub> cells and serum free T<sub>3</sub> or free T<sub>4</sub> was revealed in Graves' disease patients. Additionally, follow-up analysis indicated that in some Graves' disease patients the percentage of circulating T<sub>fh</sub> cells decreased after treatment. Furthermore, a certain number of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells together with enhanced expression of IL-21 and Bcl-6 mRNA were detected in thyroid tissues from Hashimoto's thyroiditis patients.

**Conclusion:** The current study discovered an increased frequency of T<sub>fh</sub> cells in AITD patients, which implies that this cell subset might play an important role in the pathogenesis of AITD. (*J Clin Endocrinol Metab* 97: 943–950, 2012)

**H**uman autoimmune thyroid disease (AITD), mainly including Graves' disease (GD) and Hashimoto's thyroiditis (HT), is an organ-specific immune disease characterized by autoreactive antibodies, such as anti-TSH receptor (TSHR), anti-thyroglobulin (Tg), and anti-thyroperoxidase (TPO) antibodies (Ab) (1, 2). As known, AITD is a multifactorial process involving autoimmune response and genetic and environmental influences. The antithyroid immune response begins with activation of antigen-specific helper T

cells, which could result from cross-reacting antigen. Once helper T cells are activated, they will induce B cells to secrete antithyroid autoantibodies. The anti-TSHR Ab is a thyroid-stimulating antibody that causes Graves' hyperthyroidism, whereas the anti-Tg and anti-TPO Ab in HT patients are associated with lymphocytic inflammation, thyroid gland damage, and thyroid dysfunction (2, 3).

CD4<sup>+</sup> helper T cells can regulate immune response and play a pivotal role in autoimmune reaction via secreting

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Abbreviations: Ab, Antibody; AITD, autoimmune thyroid disease; FT3, free T<sub>3</sub>; FT4, free T<sub>4</sub>; GC, germinal center; GD, Graves' disease; HT, Hashimoto's thyroiditis; ICOS, inducible costimulator; mAb, monoclonal Ab; PBMC, peripheral blood mononuclear cell; PD-1, programmed death-1; PE, phycoerythrin; PHA, phytohemagglutinin; T<sub>fh</sub>, follicular helper T; Tg, thyroglobulin; TMC, thyroid mononuclear cell; TPO, thyroperoxidase; TSHR, TSH receptor.

specific cytokines. Three major subsets—Th1, Th2, and Th17 cells—have been defined and produce interferon- $\gamma$ , IL-4, and IL-17, respectively (4–6). Recently, follicular helper T (Tfh) cells have been described as a new subset regulating the development of antigen-specific B-cell immunity. Tfh cells can migrate to the germinal center (GC) through chemokine receptor CXCR5. The expression of CXCR5 is required not only for Tfh cell migration, but also for its activity such as isotype switch; furthermore, GC formation could be impaired in the absence of CXCR5 (7, 8). Tfh cells participate in regulating the evolution of effector and memory B-cell responses as well as the production of appropriate antibodies (9). Secretion of high levels of IL-21 is a critical characteristic of Tfh cells. IL-21 or IL-21 receptor deficiency impacts the development of B cell-mediated immunity with impaired isotype switch and deficient GC development (10, 11). Antibody production by B lymphocytes requires “help” from Tfh cells in the form of cytokines and many surface molecules also. The CD28 family members, programmed death-1 (PD-1) and inducible costimulator (ICOS), are just two distinguishing molecules closely related to the function of Tfh cells (8, 12, 13). ICOS-deficient humans and mice have severe reduction of CD4<sup>+</sup>CXCR5<sup>+</sup> T cells in both GC and peripheral blood (15). ICOS/ICOS ligand-deficient mice have marked deficits in antibody production, isotype switch, and GC formation (16–18). PD-1 molecule has been implicated as a negative molecule that up-regulated on chronically activated T cells (8, 19). Previous studies have indicated that GC Tfh cells highly express PD-1 molecule (8, 20–24). Bcl-6 is an essential transcriptional factor that is characteristically expressed in Tfh cells but not other effector Th cells. Studies showed that Bcl-6 directed Tfh cell differentiation and controlled the Tfh cell signature, meanwhile, the molecule repressed transcriptional regulators of other Th cells (25).

Recent studies defined CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells as circulating Tfh cells and have shown that circulating Tfh cells increased in some patients with systemic lupus erythematosus and systemic sclerosis (26). Although AITD is a complicated disease, autoantibody production is a common and consistent characteristic in patients. Moreover, it is not yet known whether Tfh cells exert effects in the pathogenic process of AITD or not. Therefore, we sought to explore the role of Tfh cells in patients with AITD.

## Patients and Methods

### Individuals and samples

Sixty-five patients with AITD, including 36 with GD and 29 with HT, were included in the study. The diagnosis was based on

**TABLE 1.** Clinical features of AITD patients included in the study

	GD	HT	Range
n	36	29	
Gender (M/F)	10/26	3/26	
Age (yr)	38.25 ± 14.12	43.41 ± 14.37	
FT3 (pmol/liter)	9.186 ± 6.864	4.113 ± 1.156	3.10–6.80
FT4 (pmol/liter)	23.11 ± 16.52	11.35 ± 4.709	12.00–22.00
TSHR-Ab (IU/ml)	24.21 ± 6.065	12.30 ± 2.170	<15
Tg-Ab (IU/ml)	45.00 ± 24.09	523.7 ± 889.3	<30
TPO-Ab (IU/ml)	95.55 ± 217.8	242.3 ± 375.8	<10

Data correspond to the arithmetic mean ± sd. M, Male; F, female.

commonly accepted clinical and laboratory criteria. Peripheral blood samples were obtained from all patients. Main clinical data of these patients are shown in Table 1. All of the GD patients have a lower level of TSH; the level in 15 patients was 0.01  $\mu$ IU/ml or less (normal range, 0.27–4.20  $\mu$ IU/ml). Nine HT patients have a higher level of TSH, and others have a normal level. The serum concentrations of anti-TSHR, anti-Tg, and anti-TPO Ab were measured by chemiluminescent immunoassay (MAGLUMI 2000 PLUS, Shenzhen New Industries Biomedical Engineering Co., Shenzhen, China) according to the manufacturers' protocol. Patients on methimazole therapy received 20–30 mg/d for the first phase, and the dose was reduced to 5–15 mg when patients achieved a remission. Patients treated with propylthiouracil took 300–500 mg/d for the first phase and 25–100 mg for maintaining remission. All of the patients received more than 6 months of therapy. Thirty age- and sex-matched healthy subjects were included as controls—namely, 23 females and 7 males, ranging from 32 to 50 yr old. All of the control subjects were free of a history of thyroid or autoimmune diseases.

Thyroid glands were obtained from five HT patients who were undergoing thyroidectomy. All of them were positive for Tg-Ab and TPO-Ab and had normal hormone levels except for one patient (FT4, 7.92 pmol/liter). Two of the patients were bilateral goiter; others were unilateral. Lymphocytic infiltration was detected in the goiters. Thyroid tissue from three patients with simple goiter was used as the control.

All samples were taken in accordance with the regulations and approval of the Affiliated People's Hospital of Jiangsu University.

### Cell isolation and purification

Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation over Ficoll-Hypaque solution. CD4<sup>+</sup> T cells were purified from PBMC by biotin-conjugated antihuman CD4 monoclonal Ab (mAb) and antibiotin microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions.

Thyroid specimens were minced and then digested with collagenase II (Sigma-Aldrich, St. Louis, MO) for 1–2 h at 37 C and then isolated by density-gradient centrifugation over Ficoll-Hypaque solution. Finally, thyroid mononuclear cells (TMC) were obtained. Cell viability was more than 95%.

### Flow cytometric analysis

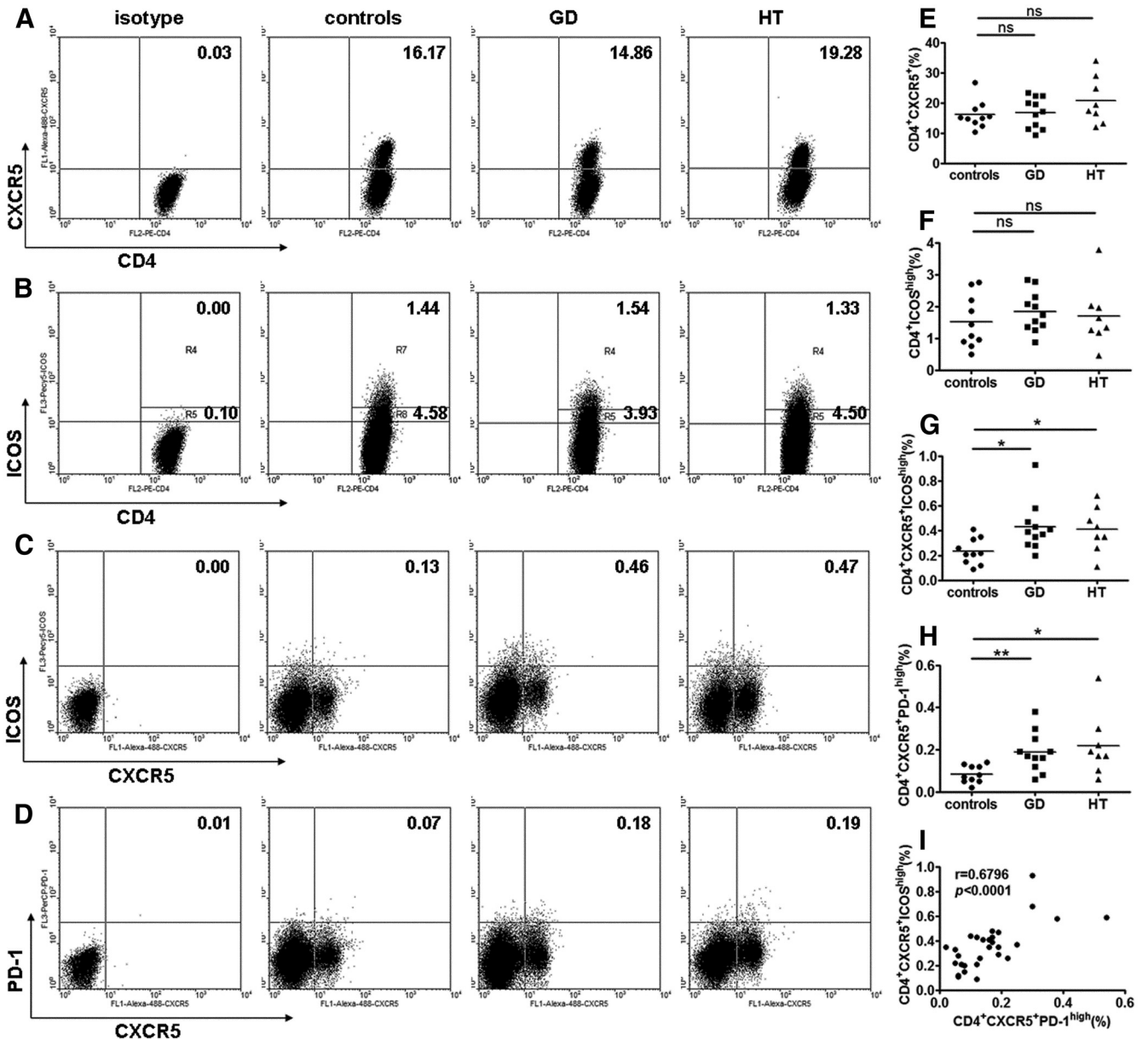
Cells were washed and immunostained with allophycocyanin-conjugated anti-CD3, phycoerythrin (PE)-conjugated anti-

CD4, phycoerythrin-cy5-conjugated anti-ICOS (eBioscience, San Diego, CA), peridinin chlorophyll protein-conjugated anti-PD-1 (Biolegend, San Diego, CA), Alexa Fluor 488-conjugated anti-CXCR5 (Becton Dickinson, San Jose, CA), or fluorescein isothiocyanate-conjugated anti-CXCR5 (R&D Systems, Minneapolis, MN) mAb against human cell surface. Isotype-matched Ab controls were used in all procedures. All the staining was performed according to manufacturers' protocol. The stained cells were then analyzed using a FACSCalibur flow cytometer and CELLQUEST software (Becton Dickinson, Sparks, MD).

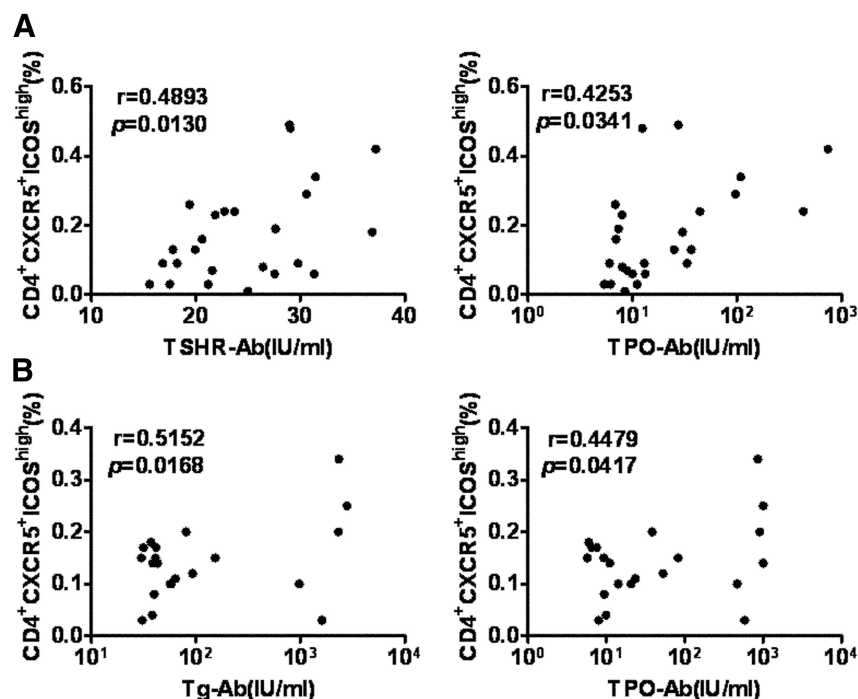
**RNA isolation and real-time PCR**

For the detection of cytokine IL-21, CD4<sup>+</sup> T cells were incubated in complete RPMI 1640 culture medium in the presence of 50 ng/ml phytohemagglutinin (PHA; Sigma-Aldrich). After 12 h of culture at 37 C under 5% CO<sub>2</sub>, cells were collected and centrifuged at 500 × g for 5 min. Then supernatant was used to test cytokine IL-21 by ELISA, and cells were used to quantify the expression of IL-21 mRNA by real-time PCR.

TRIzol reagent (Invitrogen, Carlsbad, CA) was added to CD4<sup>+</sup> T cells or TMC. Total RNA was isolated, and reverse



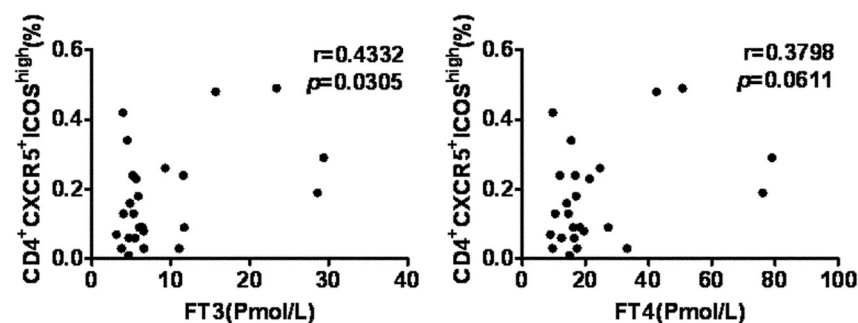
**FIG. 1.** Increased percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> and CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>high</sup> T cells in the peripheral blood of patients with AITD. Peripheral blood was obtained from 11 GD patients, eight HT patients, and 10 healthy controls. A–D, Representative dot plots of CD4<sup>+</sup>CXCR5<sup>+</sup> (A), CD4<sup>+</sup>ICOS<sup>high</sup> (B), CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> (C), and CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>high</sup> (D) T cells are shown. A, Values in the upper right quadrant correspond to the percentage of CD4<sup>+</sup>CXCR5<sup>+</sup> T cells. B, ICOS expression was determined; the upper right gate = ICOS<sup>high</sup>, the center right gate = ICOS<sup>intermediate</sup>. Values in each gate are the percentage of viable lymphocytes. C, Values in the upper right quadrant correspond to the percentage of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells. D, Values in the upper right quadrant correspond to the percentage of CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>high</sup> T cells. We used isotype controls to determine the positive cells, and all the values are gated on the CD3<sup>+</sup>CD4<sup>+</sup> cells. E–H, Percentages of CD4<sup>+</sup>CXCR5<sup>+</sup> (E), CD4<sup>+</sup>ICOS<sup>high</sup> (F), CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> (G), and CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>high</sup> (H) T cells were compared among GD and HT patients and healthy controls. I, The correlation between the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> and CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>high</sup> T cells from 11 GD patients, eight HT patients, and 10 healthy controls. Each data point represent an individual subject; horizontal lines show the mean. \*, P < 0.05; \*\*, P < 0.01. ns, No significant differences.



**FIG. 2.** The correlation between the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells in PBMC and serum levels of anti-TSHR-Ab, TPO-Ab, and Tg-Ab in AITD patients, respectively. A, The correlation between the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells and levels of anti-TSHR-Ab (left) and TPO-Ab (right) in 25 GD patients. B, The correlation between the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells and levels of anti-Tg-Ab (left) and TPO-Ab (right) in 21 HT patients.

transcription was performed according to the manufacturer's instruction (Toyobo, Osaka, Japan). Real-time PCR was performed in duplicate using Bio-Rad SYBR green super mix (Bio-Rad, Hercules, CA). Primer sequences were as follows: IL-21, sense, 5'-CACAGACTAACATGCCCTTCAT-3'; antisense, 5'-GAATCTTCACTTCCGTGTGTTCT-3', Bcl-6, sense, 5'-AAGGCCAGTGAAGCAGAGA-3'; antisense, 5'-CCGATAGGCCATGATGTCT-3'. Each gene was normalized to  $\beta$ -actin with the following primers: sense, 5'-CAGAACTACTTCAACTCC-3'; antisense, 5'-CATACTCCTTGCTTGCTGATC-3'. Data were analyzed by Bio-Rad CFX Manager software.

In the case of TMC, amplified products were electrophoresed on 2% agarose gel (Invitrogen), stained with ethidium bromide, and visualized with UV transilluminator. Finally, levels of grayscale were tested by LANE 1D software (Beijing Sage Creation Science Company, Beijing, China).



**FIG. 3.** The correlation between the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells in PBMC and FT3 (left)/FT4 (right) in 25 GD patients.

## Immunofluorescent staining and confocal microscopic images

For immunofluorescent analysis, paraffin sections of human thyroid glands were incubated with PE-anti CD4 mAb and Alexa Fluor 488-conjugated anti-CXCR5 or matched isotype controls. After washes in PBS, the sections were incubated with Hoechst 33342 for labeling nuclei. A confocal fluorescence-inverted microscope (Leica Microsystems, Wetzlar, Germany) was used for imaging of the sections. The excitation wavelength of 488 nm and two emission wavelengths of 505–550 nm and more than 560 nm were used for CXCR5 (Alexa Fluor 488, green) and CD4 (PE, red), respectively. In addition, UV was used to detect nuclei (Hoechst, blue). Images were analyzed by LAS AF software (Leica Microsystems, Wetzlar, Germany).

## Statistical analysis

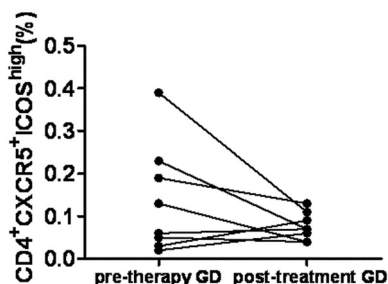
One-way ANOVA analysis was performed to determine whether there was an overall statistically significant change among the groups and posttest comparison was carried out using the Bonferroni's test. Student's unpaired or paired *t* test was performed as appropriate. Correlations between variables were determined by Spearman's correlation coefficient. Data were analyzed with GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA).

## Results

### Increased percentages of circulating CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> and CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>high</sup> T cells in AITD patients

First, we gated on CD3<sup>+</sup>CD4<sup>+</sup> T cells in PBMC and identified CXCR5<sup>+</sup> T cells and ICOS<sup>high</sup> T cells to distinguish Tfh cells from activated T cells in the peripheral blood (Fig. 1, A and B). The frequencies of CD4<sup>+</sup>CXCR5<sup>+</sup> T cells and CD4<sup>+</sup>ICOS<sup>high</sup> T cells were not significantly different in GD or HT patients compared with healthy controls (Fig. 1, E and F).

Subsequently, we compared the AITD patients with healthy controls and found significantly increased percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells in the peripheral blood from both GD and HT patients; between these two groups, there was no significant difference (Fig. 1, C and G). Due to the importance of PD-1, measurement was taken on the individual patient at the



**FIG. 4.** The percentage changes of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells in PBMC between pretherapy and posttreatment groups of eight GD patients.

same time. Reanalysis of circulating Tfh cells defined as CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>high</sup> in GD or HT patients and controls yielded results similar to those from analysis for CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells. The percentage of CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>high</sup> T cells was significantly increased in GD or HT patients compared with healthy controls (Fig. 1, D and H). Moreover, a strong correlation has been found between the ICOS<sup>high</sup> and PD-1<sup>high</sup> cells (Fig. 1I).

**High levels of autoantibodies and thyroid hormones with increased circulating CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells in AITD patients**

In patients with GD, anti-TSHR-Ab is a typical autoantibody that binds to TSHR, thereby stimulating synthesis and secretion of thyroid hormone and thyroid growth. anti-TPO-Ab is also a critical antibody because about 80% of GD patients are positive regarding this parameter (1, 3). As shown in Fig. 2A, a positive correlation was found between the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells and anti-TSHR-Ab/TPO-Ab in 25 GD patients.

Moreover, there was a positive or modest correlation between the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells and free T<sub>3</sub> (FT3) (*r* = 0.4332; *P* = 0.0305) or free T<sub>4</sub> (FT4) (*r* = 0.3798; *P* = 0.0611) in 25 GD patients (Fig. 3).

Anti-Tg-Ab frequently accompanies anti-TPO-Ab in patients with HT. The detection of either autoantibody in serum essentially correlates with the presence of thyroid lymphocytic

infiltration, and these two autoantibodies have also been used for the diagnosis of HT (1, 2, 27). Our results indicated a positive correlation between the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells and anti-Tg-Ab/TPO-Ab in 21 HT patients (Fig. 2B).

**Decreased percentage of circulating CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells in some patients with GD after treatment**

We performed follow-up analysis of eight GD patients with pretherapy and found no between-group differences in the percentage of circulating CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells compared with the group with posttreatment (treated with antithyroid drugs like methimazole and propylthiouracil for more than 6 months). However, four patients showed decreased percentages of circulating CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells (Fig. 4).

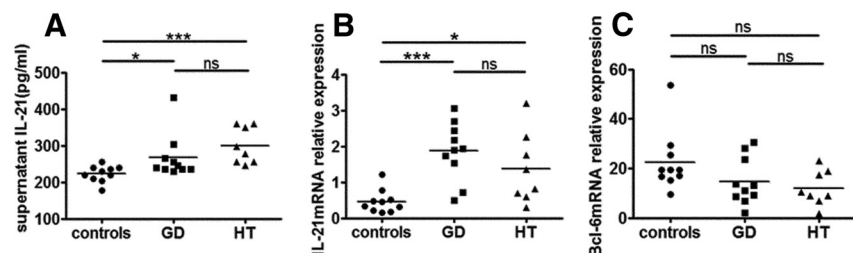
**Increased expression of IL-21 in HT or GD patients**

Recent studies showed that IL-21 cytokine and Bcl-6 transcriptional factor have critical roles in the Tfh population (11, 25). The levels of IL-21 in the culture supernatant of CD4<sup>+</sup> T cells incubated with PHA were different in HT or GD patients compared with healthy controls (Fig. 5A), and the IL-21 mRNA expression was also significantly increased in HT or GD patients (Fig. 5B). However, no significant differences were observed when Bcl-6 mRNA of CD4<sup>+</sup> T cells was detected by real-time PCR (Fig. 5C).

**Presence of Tfh cells in the thyroid tissue**

In the pathogenic process of organ-specific immune diseases, it is important for Tfh cells to recognize organ-specific autoantigens. Lymphoid infiltration, often with GC formation, is a significant feature of HT (28). Five thyroid glands of HT patients were collected, and the GC-like follicle was observed from sections stained with hematoxylin-eosin (Fig. 6A, left); CD4<sup>+</sup>CXCR5<sup>+</sup> (yellow dots) were detected by immunofluorescence microscopy (Fig. 6A, right).

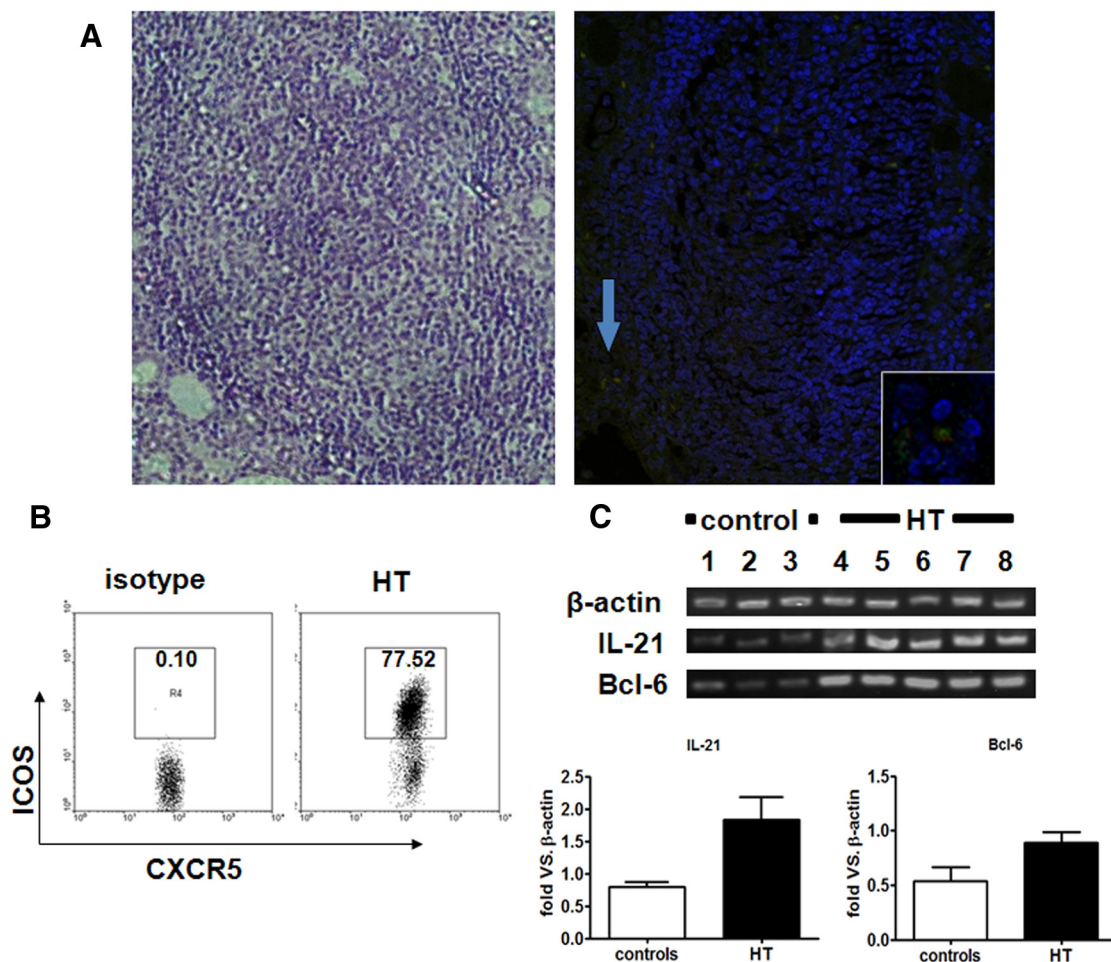
Meanwhile, flow cytometry analysis determined the expression of ICOS in the CD4<sup>+</sup>CXCR5<sup>+</sup> T cells of the TMC. As shown in Fig. 6B, CD4<sup>+</sup>CXCR5<sup>+</sup> T cells were mainly an ICOS<sup>high</sup> subset. Then, PCR analysis showed enhanced expressions of IL-21 and Bcl-6 mRNA in TMC from HT patients compared with patients with simple goiter (Fig. 6C).



**FIG. 5.** The expression of IL-21 and Bcl-6 in CD4<sup>+</sup> T cells from AITD patients. A, The supernatant levels of IL-21 in CD4<sup>+</sup> T cells pretreated with PHA were determined by ELISA from 10 GD, eight HT patients, and 10 healthy controls. B, The levels of IL-21 mRNA in CD4<sup>+</sup> T cells pretreated with PHA were detected by real-time PCR from 10 GD, eight HT patients, and 10 healthy controls. C, The levels of Bcl-6 mRNA in CD4<sup>+</sup> T cells were detected by real-time PCR from 10 GD, eight HT patients, and 10 healthy controls. Horizontal lines show the mean. \*, *P* < 0.05; \*\*\*, *P* < 0.001. ns, No significant differences.

**Discussion**

Since the discovery of AITD, which mainly includes GD and HT, research



**FIG. 6.** Tfh cells in the thyroid tissue from HT patients. A, Thyroid gland sections from HT patients were stained for CD4 (red), CXCR5 (green), and nuclei (blue). Representative merged image (20 $\times$ ) (right) and corresponding hematoxylin-eosin image (20 $\times$ ) (left) are shown. Composite green + red are the yellow dots. The arrow points to the CD4<sup>+</sup>CXCR5<sup>+</sup> cells, and the enlarged image (inset) is also shown. B, CD4<sup>+</sup>CXCR5<sup>+</sup> T cells were mainly ICOS<sup>high</sup> cells. TMC were stained for CD3, CD4, CXCR5, and ICOS, then analyzed by flow cytometry. C, Increased expression of IL-21 and Bcl-6 mRNA in TMC from five HT patients compared with three controls with simple goiter. The levels of  $\beta$ -actin, IL-21, and Bcl-6 mRNA were determined by PCR. Then, levels of grayscale were tested; corresponding values are shown below as fold vs.  $\beta$ -actin.

on the pathogenesis of the diseases has been unceasing. GD is characterized by autoantibodies, mainly TSHR-Ab (29, 30), and studies showed that there is mainly a Th2-mediated response in GD with autoantibody production and gland hyperplasia (31). Some studies have also shown that HT is a Th1-mediated autoimmune disease because there are abundant T cells infiltrating and thyrocytes are progressively impaired; however, HT is commonly diagnosed based on positive results of Tg-Ab and TPO-Ab, suggesting the importance of Th2 responses. Therefore, AITD is generally considered to be closely related to autoantibody production. Recent evidence suggested that Tfh is a main effector T-cell subset expressing B cell-promoting cytokine IL-21 (11), and this refutes the hypothesis that Th-promoted differentiation of memory B cells and generation of antibodies with high-affinity were ascribed to Th1/Th2 cells (32, 33).

Recently, a study focused on the circulating Tfh cells in systemic lupus erythematosus and systemic sclerosis patients,

and for the first time circulating Tfh cells were defined as CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells. Based on this, our study targeted AITD and investigated the Tfh cells in peripheral blood and thyroid tissues. As expected, increased CD4<sup>+</sup>CXCR5<sup>+</sup> ICOS<sup>high</sup> T cells were found in peripheral blood, and a certain number of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells infiltrated the thyroid gland, which indicated that this cell population was involved in the pathogenesis of AITD. Notably, the percentages of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells in peripheral blood from GD patients have a close correlation with FT3 or FT4; furthermore, a positive correlation between the percentage of CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells and the concentration of autoantibody was also found in both GD and HT patients. In addition, the percentages decreased after antithyroid drugs were administered to patients. Indubitably, all the above indicated that this cell subset could reflect the severity of AITD.

Besides ICOS, the PD-1 molecule is also required for cognate T-B interactions. The PD-1 molecule is considered to provide an inhibitory signal to GC Tfh cells, preventing excess CD4<sup>+</sup> T cell proliferation in a GC. Absence of PD-1 was associated with alterations in Tfh phenotypes such as reduced IL-21 (34). In our study, circulating CD4<sup>+</sup>CXCR5<sup>+</sup> T cells were analyzed for both ICOS and PD-1 in 29 subjects (10 healthy controls, 11 GD patients, and eight HT patients), and a strong correlation was found between the expression of these two cell populations, which once again indicated that the circulating CD4<sup>+</sup>CXCR5<sup>+</sup>PD-1<sup>high</sup> T cell is probably the circulating Tfh cell.

As known, the clinical manifestations of GD and HT are reversed. The former is characterized by hyperthyroidism and the later by hypothyroidism. However, the clinical course of these two disorders may fluctuate, and similar findings like lymphocytic infiltration of the thyroid as well as Tg-Ab and TPO-Ab in serum can be found in both GD and HT patients (1–3). This suggests that GD and HT may have a shared pathogenesis. Our results showed that circulating Tfh cells increased in a subset of patients with GD or HT, but a previous study indicated that Th17 cells increased in the blood in these two diseases (35). Generally, Tfh cells are related to humoral immunity, and Th17 cells are certain symbols for cellular immunity; thus, the above observations lead to an assumption that GD and HT patients might be divided into two subsets: one mediated by humoral immunity, and another mediated by cellular immunity predominantly.

However, recent studies considered blood CD4<sup>+</sup>CXCR5<sup>+</sup> T cells to be the counterparts of Tfh cells. They showed that CXCR5<sup>+</sup> Th17 cells could promote IgG and, in particular, IgA secretion and CXCR5<sup>-</sup> Th17 cells cannot induce naive B cells to secrete Ig (36). It seems that Morita's studies confused our assumption. Actually, the key to this issue is the explicit definition of Tfh and Th17 cells. CXCR3 and CCR6 were used as markers to define the Th17 cells rather than IL-17 in their studies (36). Our studies chose CXCR5 and ICOS or PD-1 as the markers of circulating Tfh because they are better representative and readily measurable for the present, which is more important in clinical research. In fact, our data seem to be consistent with Morita's result in the percentage of CD4<sup>+</sup>CXCR5<sup>+</sup> T cells between patients and healthy controls: no significant increase was found in patients. And Simpson *et al.* (26) showed that the percentage of CD4<sup>+</sup>CXCR5<sup>+</sup> T cells has no positive correlation with a specific autoantibody. These results indicate that defining functional Tfh with a single marker may not be enough, and studies of Tfh and a specific antibody may be more significant in the future.

Recent studies demonstrated that IL-21 is a critical cytokine for GC formation, Tfh cell generation, and function (11, 37, 38). Blocked IL-21 was closely associated with the decrease of Ig-secreting B cells when CXCR5<sup>+</sup> tonsillar

Tfh cells were cultured with naive human B cells (39). Our study found a stronger expression of IL-21 in circulating CD4<sup>+</sup> T cells of GD or HT patients, which may be associated with the increased percentages of circulating CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> and could even explain this increased cell population. However, analysis of the Bcl-6 mRNA expression in circulating CD4<sup>+</sup> T cells yielded no differences, which is consistent with previous studies (26, 36).

The process by which Tfh cells select mutated B cells and then promote them to differentiate into memory B cells and long-lived plasma cells (40) can sometimes fall into a wrong path and generate self-reactive B cells (41). Normally, mutated self-reactive B cells that cannot receive the second signal provided by T cells are programmed to die by apoptosis (14). Once the T cells fail to discriminate self-reactive B cells from the normal antigen-specific B cells, high-affinity autoantibodies will be generated, and thereby autoimmune disease will happen. At this point, Tfh cells are a critical checkpoint to prevent autoimmune diseases.

Our results showed that there are high levels of circulating CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>high</sup> T cells in a subset of patients with GD or HT. Further exploration of this cell subset may shine more light on the pathogenesis of AITD.

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