

## RESEARCH

# Elevated thyroglobulin level is associated with dysfunction of regulatory T cells in patients with thyroid nodules

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## Abstract

**Objective:** Thyroid nodules are usually accompanied by elevated thyroglobulin (Tg) level and autoimmune thyroid diseases (AITDs). However, the relationship between Tg and AITDs is not fully understood. Dysfunction of regulatory T cells (Tregs) plays an important role in the development of AITDs. We aimed to evaluate the effects of Tg on the function of Tregs in patients with thyroid nodules.

**Methods:** Tg levels and the functions of Tregs in peripheral blood and thyroid tissues of patients with thyroid nodules from Nanjing First Hospital were evaluated. The effects of Tg on the function of Tregs from healthy donors were also assessed *in vitro*. The function of Tregs was defined as an inhibitory effect of Tregs on the effector T cell (CD4<sup>+</sup> CD25<sup>-</sup> T cell) proliferation rate.

**Results:** The level of Tg in peripheral blood correlated negatively with the inhibitory function of Tregs ( $R = 0.398$ ,  $P = 0.03$ ), and Tregs function declined significantly in the high Tg group (Tg >77 µg/L) compared with the normal Tg group ( $11.4 \pm 3.9\%$  vs  $27.5 \pm 3.5\%$ ,  $P < 0.05$ ). Compared with peripheral blood, the function of Tregs in thyroid declined significantly ( $P < 0.01$ ), but the proportion of FOXP3<sup>+</sup> Tregs in thyroid increased ( $P < 0.01$ ). High concentration of Tg (100 µg/mL) inhibited the function of Tregs and downregulated FOXP3, TGF-β and IL-10 mRNA expression in Tregs *in vitro*.

**Conclusions:** Elevated Tg level could impair the function of Tregs, which might increase the risk of AITDs in patient with thyroid nodules.

## Key Words

- ▶ thyroglobulin
- ▶ regulatory T cells
- ▶ thyroid nodule

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## Introduction

Thyroid nodules are very common; up to 65% of the general population have at least one thyroid nodule (1), but most of them have no symptoms, and life expectancy is not affected (2). Recent studies have focused mainly on the relationship between thyroid nodule and thyroid cancer (3, 4). Thyroid nodules are usually accompanied by an increase in thyroglobulin (Tg) (5, 6) and autoimmune

thyroid diseases (AITDs), especially Hashimoto's thyroiditis (HT) (7, 8). Interestingly, the levels of Tg in patients with thyroid cancer are always considerably higher than in patients with benign thyroid nodules (9, 10). Meanwhile, the incidence of HT in patients with thyroid cancer was also higher than in patients with benign thyroid nodule (11, 12, 13). Those studies implied

that there might be some interactions between elevated Tg level and HT.

Tg accounts for approximately 75–80% of the total thyroidal protein and serves as a precursor for thyroid hormones (14). Previous studies have proved that Tg could induce lymphocytic thyroiditis by elevating Tg-Ab titers in BB/Wor rats (15) by activating T cells (16). In addition, a number of studies underlined the important roles of regulatory T cells (Tregs) on the prevention of thyroiditis in animal models. Depletion of Tregs enabled the induction of thyroiditis with mouse Tg in traditionally resistant mice as well as iodide in NOD-H2h4 mice (17, 18). Furthermore, clinical studies have suggested that the numerical and/or functional impairments of Tregs were found in patients with HT (19, 20, 21, 22, 23). We questioned whether elevated Tg level could impair the function of Tregs.

Tregs are important subtypes of T cells that are involved in the modulation of the immune response and play essential roles in the prevention of autoimmune disease (24, 25). Natural Tregs (nTregs) develop in the thymus and represent approximately 5–10% of the total number of peripheral CD4<sup>+</sup> T cells (25). They are characterized by a high expression of CD25 and the transcription factor forkhead box P3 (FOXP3). Tregs can suppress the proliferation and activity of autoreactive T cells (26).

The aim of our study was to evaluate the effects of Tg on the function of Tregs and to understand the role of Tg in inducing thyroiditis in patients with thyroid nodules.

## Materials and methods

### Reagents

Human Tg protein was purchased from Fitzgerald Industries International (Acton, MA, USA) and verified to be >98% pure by SDS-PAGE. Anti-human CD3 (Cat# 555336) and anti-human CD28 (Cat# 555725) antibodies were purchased from eBioscience. Allophycocyanin (APC)-conjugated anti-human CD25 (Cat# 555434), PerCP-Cyanine5.5-conjugated anti-human CD4 (Cat# 560650), PE-conjugated anti-human CD25 (Cat# 555432) and APC-conjugated anti-human FOXP3 (Cat# 560045) antibodies were purchased from BD Biosciences (San Jose, CA, USA) and were all of mouse origin. Tg detection kits were purchased from Siemens Healthcare Diagnostics Inc.

### Patient details and laboratory methods

Blood was collected from healthy donors with normal ranges of free thyroxin (FT4), thyroid stimulating hormone (TSH), thyroid peroxidase antibody (TPO-Ab) and thyroglobulin antibody (TG-Ab) for experiment *in vitro*. Donors with thyroid nodules (evaluated by ultrasonography), autoimmune diseases, infections or those on any type of drugs were excluded. Blood and normal thyroid tissues surrounding a thyroid nodule or adenoma in patients who underwent thyroid surgery were also collected at Nanjing First Hospital; these were confirmed by pathology. The indications for operation are according to the Chinese Management Guidelines for Patients with Thyroid Nodules and Differentiated Thyroid Cancer (2012) (27). Besides the patients who underwent thyroid surgery, blood from patients with benign thyroid nodule was also collected, while the diagnoses of nodules were supported by fine-needle aspiration biopsy. Patients with thyroid nodules were excluded if they had any systemic disease or used drugs that may either affect Tg level or immune balance (e.g. infection and autoimmune diseases). The Bioethical Committee of the Nanjing First Hospital approved this study, and written informed consent was obtained from each patient and healthy donor.

Thyroid tissue was homogenized and centrifuged at 380g for 10 min. Supernatant (thyroid follicular fluid) was then collected to measure Tg levels. Blood and thyroid Tg levels were measured using chemiluminescent immunometric assays (Elecsys TG II, Roche Diagnostics GmbH) on a Modular Analytics E170 analyzer (Roche Diagnostics GmbH), and the normal reference range was 3.5–77 ng/mL as described in previous study (6). The total protein content of thyroid follicular fluid and plasma samples was measured using a BCA Protein Assay Kit (Sigma Aldrich), following the manufacturer's instructions. Thyroid volume before surgery was obtained by computing the volumes of both lobes (lobe (mL) = length (cm) × width (cm) × depth (cm) × 0.479). Nodules and/or cystic areas were included in the thyroid volume (reference values, 18 mL for females and 25 mL for male patients) (28).

### Isolation of peripheral blood and thyroid Tregs

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque density (Sigma Aldrich) gradient centrifugation. For the separation of thyroid mononuclear cells, thyroid specimens were homogenized and passed

through a 75- $\mu$ m steel mesh, and then mononuclear cells were isolated by Ficoll-Paque centrifugation. After washing twice with 10 mL of PBS, cells were re-suspended in Roswell Park Memorial Institute (RPMI)-1640 medium containing penicillin (80 U/mL), streptomycin ( $1.38 \times 10^{-4}$  mol/L), L-glutamine ( $2.05 \times 10^{-3}$  mol/L), L-glucose (0.01 mol/L) and sodium bicarbonate (0.02 mol/L), supplemented with 10% fetal bovine serum (FBS; Invitrogen).

Tregs were isolated from mononuclear cells using a human Treg cell magnetic-activated cell sorting (MACS) kit (Miltenyi Biotec). Effector T cells (Teffs, CD4+CD25<sup>-</sup> T cells) were also collected after the isolation of Tregs with CD25 antibody. After separation, cells were re-suspended in 2 mL of RPMI-1640 medium supplemented with 10% FBS. The purity of peripheral blood and thyroid Tregs was checked by flow cytometry and cell purity was consistently found to be >95%.

### Immunofluorescence staining

Thyroid tissue sections, incubated with monoclonal rabbit anti-FOXP3 (Cell Signaling Technology, dilution 1:500) were subsequently incubated for 30 min with goat anti-rabbit immunoglobulin antibodies conjugated to Alexa-Fluor 488 (DAKO). DAPI (4',6-diamino-2-phenylindole, KEYGEN, Jiangsu, CN) was used to label nuclear DNA. Appropriate isotype antibodies were used as negative controls. The labeled sections were imaged using a fluorescence microscope.

### Functional analysis of Tregs

The main function of Tregs was to inhibit Teffs proliferation. We tested the function of Tregs as described previously (23, 29): Tregs (>97% pure) were cultured with constant amount of Teffs cells at various ratios (Treg:Teffs=1:2, 1:4, and 1:8 or 1:10) in medium containing 10% FBS at 37°C under 5% CO<sub>2</sub>. Anti-human CD3 ( $5 \times 10^{-8}$  mol/L) and anti-human CD28 ( $2.27 \times 10^{-8}$  mol/L) antibodies were added to the cultures. Cells were simultaneously treated with Tg at different concentrations (0, 0.1, 1, 10, and 100  $\mu$ g/mL) and cultured for 3 days in 24-well plates ( $5 \times 10^5$  cells/well). Thyroid Tregs were also co-cultured with homologous peripheral blood Teffs. To determine cell proliferation, carboxyfluorescein succinimidyl ester (CFSE; BD Biosciences, San Jose, CA, USA) was added to the T cell suspension in an RPMI-1640 medium at a final concentration of  $5 \times 10^{-6}$  mol/L. Cells were incubated for 15 min at 37°C and then treated with APC-anti-CD25 for another 30 min at 4°C. T cell proliferation was analyzed

using FACS Canto™ II (BD Biosciences). The inhibition rate (%) was calculated using the formula: ((Teffs proliferation alone – Teffs proliferation with Treg)/Teffs proliferation alone)  $\times$  100%.

### PBMC stimulation with Tg

Isolated PBMCs were counted and cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C under 5% CO<sub>2</sub>. Different concentrations of Tg (0, 0.1, 1, 10 and 100  $\mu$ g/mL) were added; then the cells were incubated for 3 days. Similar to earlier experiments, anti-human CD3 ( $5 \times 10^{-8}$  mol/L) and anti-human CD28 ( $2.27 \times 10^{-8}$  mol/L) antibodies were also added to the wells. After 3 days, Tregs were isolated from the mononuclear cells using a human Treg cell MACS kit, and then RNA was isolated for further analysis.

### RNA isolation and real-time PCR

Cells were homogenized in TRIzol reagent (Sigma-Aldrich), and RNA extraction was performed using TaKaRa RNAiso reagent (TaKaRa). For reverse transcription, cDNA was synthesized using a PrimeScript™ RT Master Mix (TaKaRa). The real-time PCR efficiency for each primer pair was calculated using standard curves generated through serial dilution of cDNA from Tregs. Primer sequences are shown in Table 1. PCR reactions were performed using an ABI PRISM 7500 Sequence Detector (Applied Biosystems). The PCR conditions were set as follows: an initial incubation step for 30s at 95°C followed by 40 cycles of 5s at 95°C and 34s at 60°C. The normalized expression values for each transcript were calculated as the quantities of *FOXP3*, *TGF- $\beta$*  and *IL-10* mRNA relative to the quantity of  *$\beta$ -ACTIN* mRNA using the 2<sup>- $\Delta\Delta$ Ct</sup> method. All reactions were performed independently at least three times.

### Flow cytometric analysis of Treg cell phenotypes

PBMCs were treated with or without Tg (100  $\mu$ g/mL) for 3 days. Cells were then washed and stained with PerCP-Cyanine5.5-anti-CD4, PE-anti-CD25 and APC-anti-FOXP3. Cells were analyzed using a FACS Canto™ II (BD Biosciences).

### Statistical analysis

Statistical analysis was performed using SPSS 16.0 Software (SPSS, Inc.). Differences between two groups were analyzed using Student's *t*-test. The relationships

**Table 1** Primer sequences for real-time PCR.

Gene	Forward (5'-3')	Reverse (5'-3')
<i>β-ACTIN</i>	ATCTGCTGGAAGGTGGACAGCGA	CCCAGCACAATGAAGATCAAGATCAT
<i>FOXP3</i>	GTGGCCCGGATGTGAGAAG	GGAGCCCTTGTGGATGATG
<i>TGF-β</i>	AACGAACTGGCTGTCTGC	CCTCTGCTCATTCCGCTTAG
<i>IL-10</i>	GCTGGAGGACTTAAGGGTTAC	ATGTCTGGGTCTTGGTTC

*FOXP3*, Forkhead box P3; *IL-10*, interleukin-10; *TGF-β*, transforming growth factor-β.

between Tg levels in the blood and Treg cell functionality were analyzed by multiple linear regression. Differences in the functionality of Tregs from a single individual were analyzed using a paired Student's *t*-test. Results were presented as means ± s.e.m., or as median (IQR), and a *P* value < 0.05 was considered statistically significant.

## Results

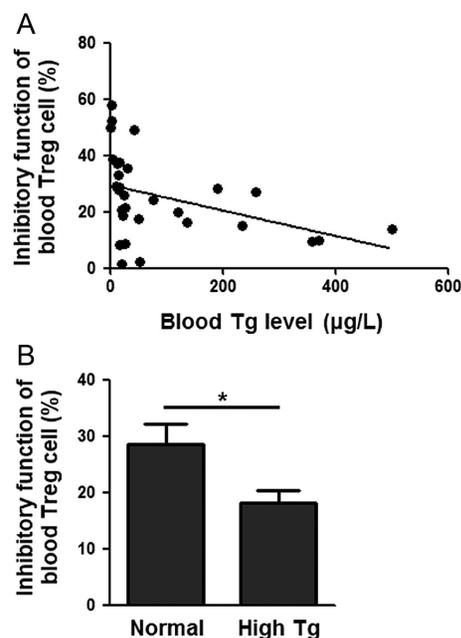
### Relationship between the functions of Tregs and Tg levels in the peripheral blood

The functions of Tregs and Tg levels in the peripheral blood of 30 patients with benign thyroid nodules were detected at the same time. Linear regression analysis showed that Tg level was negatively correlated with the inhibitory function of Tregs in the peripheral blood ( $R = -0.398$ ,  $P = 0.03$ , Fig. 1A). This association remained significant when gender; age; TSH, FT3, FT4 and TG-Ab levels; nodule size and number and thyroid volume were included in a stepwise multiple regression analysis (standardized  $\beta = -0.398$ ,  $P = 0.03$ ). We also compared the function of Tregs in the high (Tg > 77 µg/L, according to the normal reference range) and normal (Tg ≤ 77 µg/L) Tg groups and found a significantly declined Tregs function in the high Tg group ( $11.4 \pm 3.9\%$  vs  $27.5 \pm 3.5\%$ ,  $P < 0.05$ , Fig. 1B). The clinical characteristics of the patients in the two groups are shown in Table 2, and thyrotrophin receptor antibody (TR-Ab), TPO-Ab and Tg-Ab levels were slightly higher in the high Tg group than in the normal Tg group, although the levels of these antibodies in the two groups were within the normal ranges. Moreover, the nodular size and number, and the thyroid volume in the two groups were similar ( $P > 0.05$  for all, Table 2).

### Inhibitory functions and proportion of thyroid Tregs

The levels of Tg and the ratio of Tg to total protein in the thyroid follicular fluid were significantly higher than those in the peripheral blood ( $P < 0.001$ , Fig. 2A).

We found that FOXP3<sup>+</sup> Tregs were concentrated in several thyroid follicles (Fig. 2B). The proportion of FOXP3<sup>+</sup> Tregs among CD4<sup>+</sup> T cells in thyroid tissue was higher than in peripheral blood ( $P < 0.01$ , Fig. 2C). We wondered whether elevated Tg level could affect the function of Tregs in the thyroid. Thyroid Tregs were co-cultured with peripheral blood Tregs from the same patient for 3 days in different ratios (1:2, 1:4 and 1:8). The function of peripheral blood Tregs was tested as control. The results showed that the inhibitory function of thyroid Tregs was



**Figure 1**

The relationship between blood Tg levels and Treg cell function. (A) Blood Tg concentrations in 30 patients with benign thyroid nodules were measured. Tregs from these patients were co-cultured with CD4<sup>+</sup>CD25<sup>-</sup> T cells (Teffs) at a 1:10 ratio and their immunomodulatory function were determined. The inhibitory function of Tregs were analyzed using the inhibition rate (%) of Teffs proliferation and was calculated using the equation: ((proliferation of Teffs – proliferation of Teffs plus Treg)/ proliferation rate of Teffs alone) × 100%. The correlation between Tg concentration and the inhibition rate (%) of Teffs proliferation (representing Treg cell function) was analyzed by linear regression. (B) Patients were divided into high Tg group (Tg > 77 µg/L) and normal group (Tg ≤ 77 µg/L). Tregs function were compared between the two groups. \**P* < 0.05.

**Table 2** Clinical characteristics of the patients with benign thyroid nodules.

	Normal Tg group	High Tg group	P value	Normal range
<i>n</i>	21	9	-	
Gender (male, %)	23.8	22.2	1.000	-
Age (year)	49.0 ± 2.4	52.7 ± 4.3	0.430	-
TSH (mIU/L)	2.1 ± 0.2	2.0 ± 0.3	0.770	0.35–4.94
FT3 (pmol/L)	4.4 ± 0.1	4.0 ± 0.3	0.161	2.63–5.70
FT4 (pmol/L)	15.7 ± 0.4	15.5 ± 1	0.792	9.0–19.0
TR-Ab (IU/L)	<0.3	0.3 (0.4, 0.5)	-	<1.75
TPO-Ab (IU/L)	5.0 (5.0, 7.7)	7.1 (5.0, 12.7)	0.255	<34.0
Tg-Ab (IU/mL)	11.2 (10.0, 20.4)	21.3 (11.3, 24.9)	0.095	<115.0
Nodular size (mm)	17.9 ± 4.1	20.9 ± 6.8	0.695	-
Nodular number	1.8 ± 0.1	1.8 ± 0.1	0.928	-
Thyroid volume (mL)	11.00 ± 1.2	38.59 ± 23.45	0.274	Females <18 mL Male <25 mL

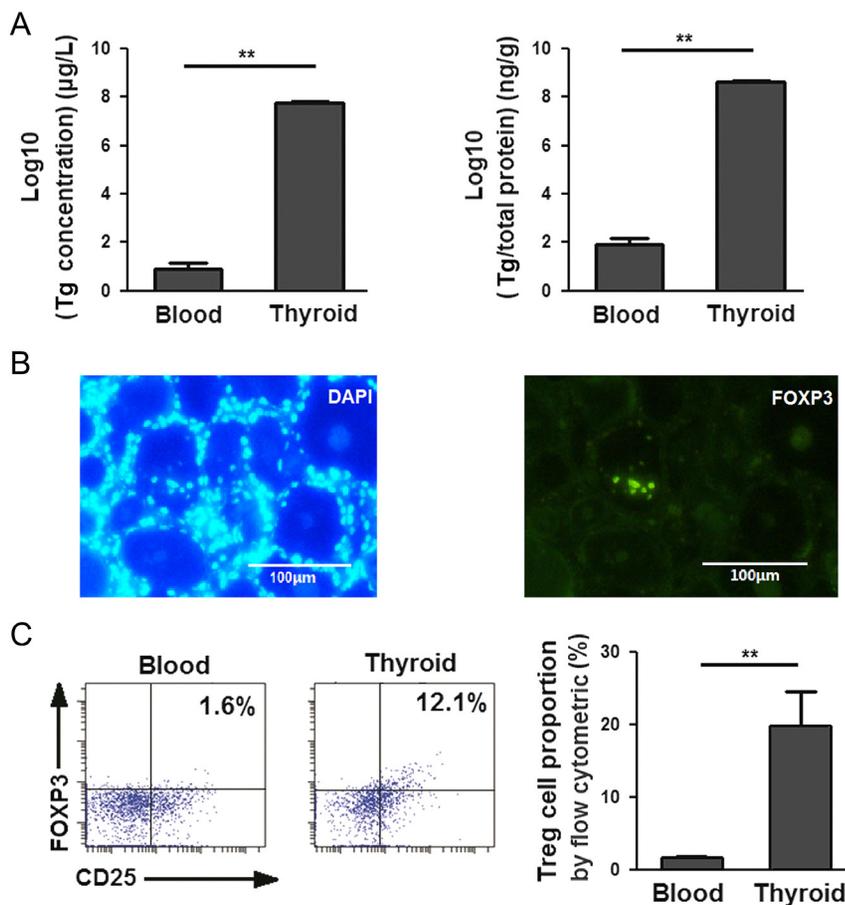
FT3, free triiodothyronine; FT4, free thyroxine; *n*, number; Tg, thyroglobulin; Tg-Ab, thyroglobulin antibody; TPO-Ab, thyroid peroxidase antibody; TR-Ab, thyrotrophin receptor antibody; TSH, thyroid-stimulating hormone.

weaker than that of the peripheral blood Tregs ( $P < 0.01$ , Fig. 3A and B).

### Effects of Tg on the function of Tregs

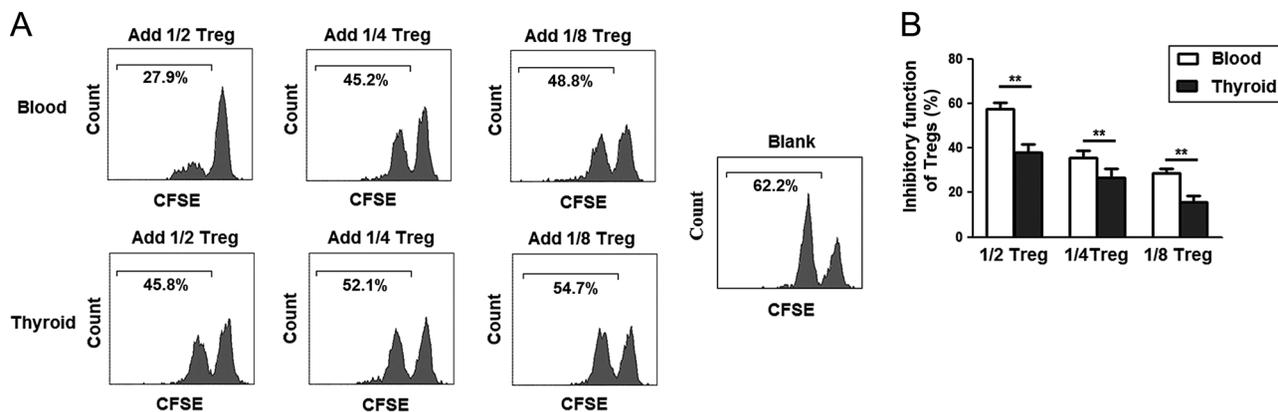
To determine the impact of Tg on the function of Tregs, Tregs and Teffs from peripheral blood of healthy donors

were co-cultured for 3 days in various ratios (1:2, 1:4 and 1:8) with different concentrations of Tg (Fig. 4A). We found that a high concentration of Tg (100 µg/mL) significantly suppressed the inhibitory function of Tregs compared with control ( $P < 0.05$ ). Conversely, low concentration of Tg (1 µg/mL) promoted the inhibitory function of Tregs compared with control ( $P < 0.01$  with



**Figure 2**

TG and Tregs in thyroid tissue. Thyroid tissues were taken from seven patients who had undergone surgery for benign thyroid nodules. The concentrations of Tg (A) and the ratio of Tg/total protein in the thyroid follicular fluid and peripheral blood were measured by chemiluminescent immunometric assay. (B) Immunofluorescence microscopy of normal thyroid sections. DAPI was used to label nuclear DNA and FOXP3 was used to label Tregs. Original magnification ×200. (C) Thyroid mononuclear cells were stained with anti-CD4-PerCP-Cyanine5.5, anti-CD25-PE, and anti-FOXP3-APC, as well as PBMCs. The proportion of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> cells in the CD4<sup>+</sup> T cell population was determined by flow cytometry. The experiment was performed seven times independently. \*\* $P < 0.01$ .

**Figure 3**

Suppressive functionality of thyroid Tregs. (A) Thyroid tissues were taken from six patients who had undergone surgery for benign thyroid nodules. Thyroid Tregs were co-cultured with peripheral blood effector T cells (Teffs, CD4<sup>+</sup>CD25<sup>-</sup> T cells) at different ratios (1:2, 1:4, and 1:8) for 3 days. Teffs were labeled with CFSE and analyzed by flow cytometry. (B) The inhibitory function of Tregs were analyzed using the inhibition rate (%) of Teffs proliferation and was calculated using the equation: ((proliferation of Teffs – proliferation of Teffs plus Treg)/proliferation rate of Teffs alone) × 100%. The experiment was performed independently six times.

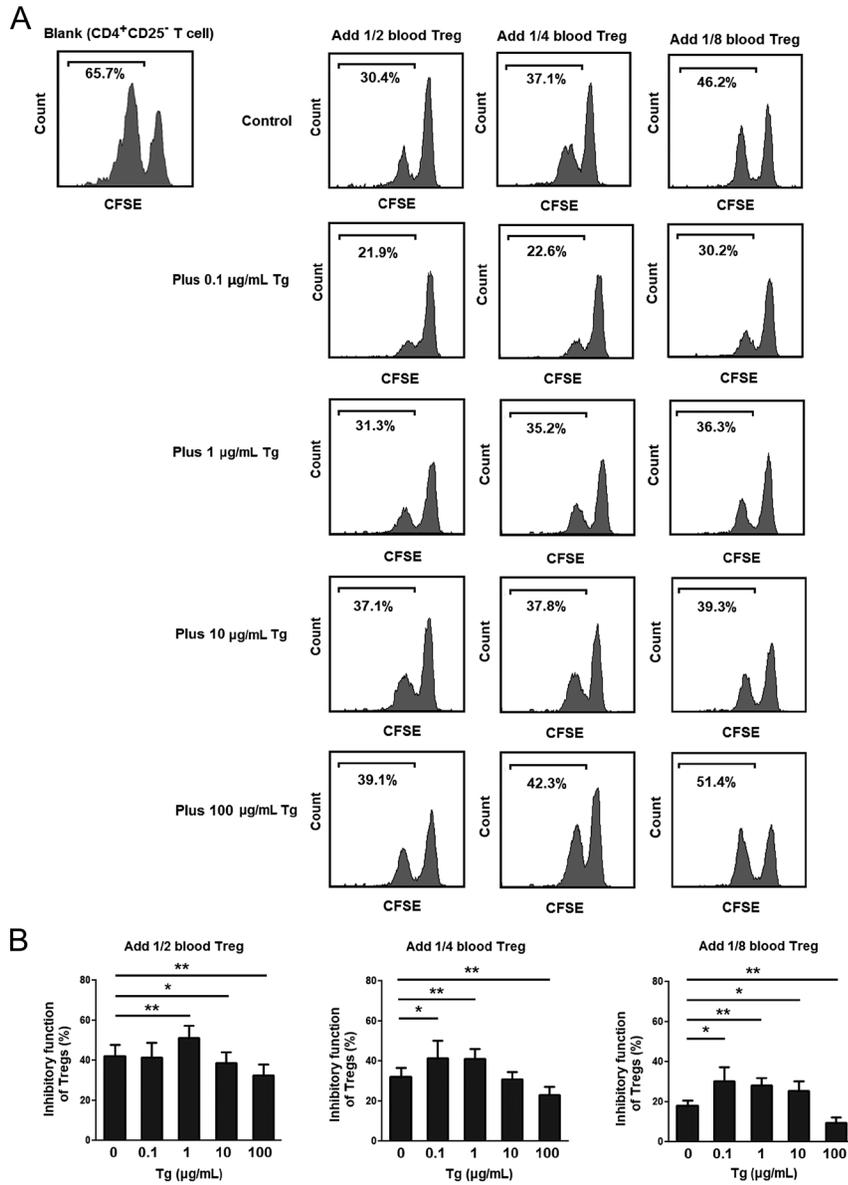
Treg/Teff=1/2 and  $P<0.05$  with Treg/Teff=1/4 and 1/8, Fig. 4B). In order to further investigate the underlying mechanisms of Tg on the function of Tregs, we isolated Tregs from peripheral blood of healthy donors and treated them with different concentrations of Tg (0, 0.1, 1, 10 and 100 µg/mL) for 3 days. We found that a low concentration of Tg (1 µg/mL) significantly stimulated the mRNA expression of *FOXP3*, *TGF-β* and *IL-10*;  $P$  for all was  $<0.01$  (Fig. 5). However, higher concentrations of Tg (100 µg/mL) inhibited the mRNA expression of *FOXP3*, *TGF-β* and *IL-10* ( $P<0.01$ ) (Fig. 5). To examine the direct effect of Tg on Teffs proliferation, we also added 100 µg/mL Tg to Teffs without Treg. The proliferation rate of Teff in 100 µg/mL Tg group and blank group were  $56.2\pm 6.8\%$  vs  $65.7\pm 9.1\%$ , respectively,  $P=0.055$ .

## Discussion

In the present study, we found a correlated dysfunction of Tregs with Tg level in patients with benign thyroid nodules and a significantly decreased function of Tregs in the high Tg group (Tg  $>77$  µg/L) compared with the normal Tg group. Serum Tg levels could be elevated in most proliferative thyroid diseases (5, 30), and the numerical and/or functional impairments of Tregs have been shown to be involved in the pathogenesis of AITDs in humans (18, 23, 31, 32). The mechanism of this high incidence of AITDs might be due to the high level of Tg, which impairs the function of Tregs in thyroid nodules and cancer.

We also confirmed that high levels of Tg could affect the function of Tregs *in vitro*, with decreased FOXP3, IL-10 and TGF-β expressions. FOXP3 was not only a molecular marker and cell lineage specification factor for Tregs, but also critical for the regulatory activity of Tregs (33). Higher levels of FOXP3 expression suppresses the activity of non-regulatory T-cells in rodents (34). In humans, mutations in the *FOXP3* gene are linked to Tregs deficiency, causing a severe systemic autoimmune disease called immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome (35). IL-10 and TGF-β are soluble factors that are expressed by Tregs, and they have direct suppressive effects on Teffs (36, 37). High levels of Tg affecting the function of Tregs might be due to the inhibitory effects of Tg on the expression of FOXP3, IL-10 and TGF-β in Tregs. Inversely, low levels of Tg could improve the function of Tregs and stimulate the mRNA expression of FOXP3, TGF-β and IL-10. Previous studies suggested that 100% CBA/J mice developed thyroiditis with positive Tg-Ab when high concentration of Tg (100 µg) was injected *i.v.* once (17), but only 17% of the mice developed thyroiditis after low concentration of Tg (40 µg daily) injection for 10 days (38). None of the mice developed thyroiditis when they were injected with the low concentration of Tg (20 µg daily) and TSH (0.25 IU daily) (38). The results of these studies might be due to the effects of the different concentrations of Tg on Tregs.

Although high concentrations of Tg suppressed Treg cell function, low concentrations had the opposite effect *in vitro*, suggesting that Tg may act differently to

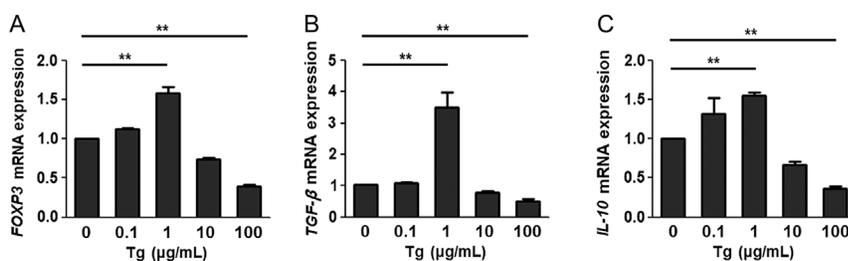


**Figure 4**

The bidirectional effects of Tg on Treg cell function. (A) Tregs were isolated from peripheral blood of healthy donors and co-cultured with CD4<sup>+</sup>CD25<sup>-</sup> T cells labeled with CFSE at different ratios (1:2, 1:4 and 1:8), and simultaneously treated with TG at various concentrations (0, 0.1, 1, 10 and 100 µg/mL) for 3 days. Anti-human CD3 ( $5 \times 10^{-8}$  mol/L) and anti-human CD28 ( $2.27 \times 10^{-8}$  mol/L) antibodies were also added to the cultures. The proliferation rates of Teffs were analyzed by flow cytometry. (B) Inhibitory function of Tregs, represented by the inhibition rate (%) on Teffs proliferation, was calculated using the equation: ((proliferation of Teffs – proliferation of Teffs plus Treg)/proliferation rate of Teffs alone) × 100%. The experiment was performed independently 11 times. \* $P < 0.05$ , \*\* $P < 0.01$ .

regulate Treg cell function depending on whether it is in the thyroid tissue or peripheral blood. The bidirectional effects of Tg on Treg cells were similar to the effects of Tg on the growth of the thyroid follicular epithelial cells (39, 40). The induction of thyroid cell growth was

noted only at lower concentrations of Tg, whereas the inhibition of cell growth and follicular function were induced dose dependently at higher concentrations of Tg. Suzuki *et al.* suggested that different recognition systems, rather than the two different domains of the Tg protein,



**Figure 5**

Effects of Tg on FOXP3 and cytokine expression in Tregs. PBMCs were isolated from peripheral blood of healthy donors and incubated with different concentrations of Tg (0, 0.1, 1, 10 and 100 µg/mL) for 3 days. Then, Tregs were isolated by MACS, and the mRNA expression levels of (A) FOXP-3, (B) TGF-β and (C) IL-10 were determined by real time-PCR. The experiment was performed at least three times independently. \*\* $P < 0.01$ .

are responsible for this biphasic activity in thyroid cell growth (39).

Tg is produced exclusively by the follicular cells of the thyroid and the concentration of Tg in thyroid is much higher than that in peripheral blood, especially in patients with thyroid nodules. The present study showed that the immune regulatory function of Tregs in thyroid tissue was much lower than in peripheral blood. Only a small number of individuals are prone to developing AITDs among patients with thyroid nodules. There must be a mechanism to maintain the immune homeostasis in thyroid. Although the function of Tregs decreased, the proportion of Tregs increased in thyroid. The increased proportion of Tregs might have compensated for the decrease in Tregs function. Certain factors, such as gene defects, virus infection, iodine intake, stress or destruction of thyroid follicles caused by thyroid nodules (such as patients in high Tg group in the present study) and cancer, can lead to a raise of Tg level (30, 41, 42). We speculate that if the concentration of Tg is further increased to levels higher than those can be balanced by compensatory mechanism in some patients with thyroid nodules, the immune homeostasis may malfunction and the AITDs might develop.

In conclusion, for the first time, we found that a high level of Tg could impair the function of Tregs in patients with thyroid nodules, which could explain the high incidence of HT in such patients. The mechanisms of the dysfunction of Tregs might be due to the inhibitory effects of Tg on the expression of FOXP3, TGF- $\beta$  and IL-10. The present study provides a new insight to the management of thyroid nodules, especially those occurring with elevated Tg levels.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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