RESEARCH

Expression of thyroglobulin by regulatory T cells in thyroid tissue

Yun-Qing Zhu^{1,*}, Yun Hu^{1,*}, Ke He², Na Li¹, Peng Jiang³, Yu-Qin Pan⁴, Hong Zhou⁵ and Xiao-Ming Mao¹¹Department of Endocrinology, Nanjing First Hospital, Nanjing Medical University, Nanjing, China²Department of Endocrinology, Wuxi Traditional Chinese Medicine Hospital, Wuxi, China³Department of Thyroid and Breast Surgery, Nanjing First Hospital, Nanjing Medical University, Nanjing, China⁴Central Laboratory, Nanjing First Hospital, Nanjing Medical University, Nanjing, China⁵Department of Immunology, Nanjing Medical University, Nanjing, ChinaCorrespondence should be addressed to X-M Mao: maoxming@163.com

(*Y-Q Zhu and Y Hu contributed equally to this work)

Abstract

The follicles are the minimal functional unit of the thyroid; the morphology and the function of each follicle can vary significantly. However, the reasons for the apparent follicular heterogeneity are poorly understood. Some tissue-resident regulatory T cells (Tregs) have a special phenotype that expresses unique molecules related to local tissue and regulates the tissue functions. The aim of this study was to identify the phenotype of thyroid Tregs and the roles of thyroid Tregs in thyroid physiological regulation. Thyroid tissue and peripheral blood samples were obtained from patients with benign thyroid nodules. Microarray-based gene expression, flow cytometry, immunofluorescence microscopy, and functional analysis of thyroid Tregs were performed. Here, we demonstrated that human thyroid Tregs expressed high level of thyroglobulin (Tg), both gene and protein. The immunofluorescence microscopy of thyroid section showed that the FOXP3⁺Tg⁺ cells concentrated in some of the thyroid follicles, at the side of the thyroid follicle. The peripheral blood Tregs expressed minimal levels of Tg, and low levels of Tg could effectively induce peripheral blood Tregs to express Tg, which was independent of thyrotropin simulation. Furthermore, the Tg secreted freely from thyroid Tregs that negatively regulated some thyroid-related genes expression. Our results revealed that the thyroid Tregs was a distinct population of Tregs, which expressed high level of Tg. The thyroid Tregs regulate thyroid function by Tg that is paracrine from the cells.

Key Words

- thyroid
- thyroglobulin
- thyroid follicles
- thyroid function

*Journal of Molecular
Endocrinology*
(2019) **63**, 261–271

Introduction

Regulatory T cells (Tregs) are known to be diverse populations of lymphocytes that act as a key regulator involved in mediating the homeostasis of peripheral tolerance in higher vertebrates (Josefowicz *et al.* 2012). Tregs originate from the thymus where they interact with thymus-resident antigen-presenting cells – an instructive cytokine milieu stimulates the T cell receptor (TCR) and

leads to the selection into the Tregs lineage that expresses *FOXP3* gene. Once mature, Tregs leave the thymus and migrate into either the secondary lymphoid tissues, that is, lymph nodes and spleen, or the peripheral non-lymphoid tissues. Although Tregs comprise only 3–5% of the peripheral blood T cell pool, they play important roles in regulating the immune system activation and preventing

the pathological reactivity to self (autoimmunity) and/or harmless antigens (allergy) (Richards *et al.* 2015).

Apart from FOXP3, they are also characterized by the expression of a variety of co-stimulatory and co-inhibitory molecules, like CD28, cytotoxic T lymphocyte antigen 4 (CTLA-4), inducible co-stimulator, tumor necrosis factor (TNF)/TNF receptor family members (RANKL, GITR, OX40, 4-1BB, etc.), chemokine, Toll-like receptors, and a variety of immunosuppressive cytokines (interleukin (IL)-10, transforming growth factor (TGF)- β , IL-35, etc.) (Lu & Rudensky 2009, Shevach 2009, Campbell & Koch 2011, Chen & Oppenheim 2011, Ohkura *et al.* 2011).

Recently, increasing evidence in the literature has suggested that some tissue-resident Tregs can express some special molecules relating to the local tissues; for example, fat tissue Tregs express peroxisome proliferator-activated receptor gamma that generally is expressed in adipose cells, and skeletal muscle tissue Tregs express amphiregulin that generally is expressed in skeletal muscle cells (Cipolletta *et al.* 2012, Burzyn *et al.* 2013a). Apart from the classical regulation of immune responses, these Tregs have several functional roles in the local tissues (Burzyn *et al.* 2013b, Cipolletta 2014). The Tregs of fat tissue could control the inflammatory state and restore insulin sensitivity in obese mice (Cipolletta *et al.* 2012) and the Tregs of the skeletal muscle potentiate muscle repair in mice with acutely injured skeletal muscle (Burzyn *et al.* 2013a). These tissue-resident Tregs express most of the canonical Tregs signature, retain conventional Tregs markers, such as FOXP3, CD25, CTLA-4, GITR and OX40, and maintain the traditional immune suppression function (Feuerer *et al.* 2009). However, the prevalence, transcriptome, and TCR repertoire of this population are all distinct from those of their counterparts in lymphoid organs. These studies have suggested that Tregs could remodel in a variety of tissues, exhibiting particular features and functions associated with local tissues.

The thyroid gland is an important endocrine organ. The main function of the thyroid is synthesis and secretion of thyroid hormones (THs, T₃ and T₄). The thyroid gland consists of thyroid follicles, which constitute the minimal functional unit of the thyroid. The thyroid follicle is enclosed by an independent basket-like plexus of anastomotic fenestrated capillaries that cover 20–50% of its surface. Despite the seemingly uniform thyroid-stimulating hormone (TSH) stimulation among follicles, the morphology and the function of each follicle can vary significantly (Imada *et al.* 1986). The follicles in the same normal thyroid tissue can have a broad distribution of size, colloid Tg concentrations, iodide uptake, TH accumulation,

enzymatic activities, and expression of thyroid-specific transcription factors (Sellitti & Suzuki 2014, Oda *et al.* 2017). This phenomenon, termed follicular heterogeneity, suggests that factors other than TSH levels in the blood are involved in regulating individual follicular function. The reasons for the apparent default of the thyroid to a condition of follicular heterogeneity have thus far been poorly understood (Sellitti & Suzuki 2014). In this study, we identified the phenotype of thyroid Tregs and explored its roles in regulating thyroid physiological function.

Materials and methods

Patients

Surgical thyroid tissues and peripheral blood samples were obtained from 49 patients (37 females and 12 males; age range, 21–65 years) who underwent surgery for benign thyroid nodule. The benign thyroid nodule was diagnosed by thyroid ultrasonography or computed tomography and technetium-99 radioisotope scans. The patients did not receive any specific treatment for thyroid disease. The levels of thyroid hormones and thyroid autoantibody were in the normal range (Table 1). The normal thyroid specimens near the thyroid nodules were obtained during the surgery. The diagnoses were confirmed postoperatively by pathological examination. Written informed consent was obtained from the subjects before the study, and ethics permission was obtained for the use of the samples, and the study was approved by the Local Ethics Committee of Nanjing Medical University.

Isolation of thyroid follicular cells and mononuclear cells

Thyroid follicular cells were prepared, as previously described (Marazuela *et al.* 1994, Garcia-Lopez *et al.* 2001). The tissues were minced to small fragments and digested by 250 IU/mL collagenase II and 0.25% trypsin (Gibco) in D-Hank's solution for 90 min at 37°C. The digested tissues were mechanically dispersed until a homogeneous suspension was obtained. After washing with D-Hank's solution, the cell suspension was filtered through a 125 μ m mesh and then cultured in DMEM medium containing 10% fetal bovine serum (Gibco), 2 mM glutamine, and 50 μ g/mL penicillin/streptomycin at 37°C and 5% carbon dioxide. The cells were used within the third to sixth passage.

Thyroid mononuclear cells were obtained from the minced thyroid tissue suspension. The thyroid tissue was rinsed in 4°C D-Hank's solution, minced, teased finely,

Table 1 Clinical characteristics in patients with euthyroid function.

	mean $\pm$ s.d.	Range	Normal value
Age (years)	45.59 $\pm$ 10.46	21–65	
Gender (male/female)	12/37		
TSH (mIU/L)	1.89 $\pm$ 0.87	0.39–3.76	0.45–4.94
FT3 (pmol/L)	4.50 $\pm$ 0.65	2.68–5.52	2.63–5.70
FT4 (pmol/L)	16.00 $\pm$ 1.67	10.14–18.48	9.00–19.0
TRAb (IU/L)	0.40 $\pm$ 0.14	0.3–0.79	<1.75
TPOAb (IU/mL)	10.99 $\pm$ 6.89	5.0–31.21	<34
TGAb (IU/mL)	34.32 $\pm$ 13.54	1.31–92.68	<115
WBC (10^9 /L)	5.59 $\pm$ 1.50	3.5–9.07	3.5–9.5
NC (10^9 /L)	3.41 $\pm$ 1.20	1.86–6.98	1.8–6.3
LC ($\times 10^9$ /L)	1.84 $\pm$ 0.50	1.18–3.18	1.1–3.2
ALT (U/L)	19.67 $\pm$ 9.58	6–49	9–50
AST (U/L)	20.51 $\pm$ 5.71	15–38	15–40

ALT, alanine aminotransferase; AST, aspartate aminotransferase; LC, lymphocyte count; NC, neutrophil count; WBC, total white blood cell count.

and resuspended in 10mL D-Hank's solution to release infiltrating mononuclear cells. The mononuclear cells in the 30mL minced tissue suspension were separated by Ficoll density centrifugation (Lymphoprep, density 1.077 g/mL, Tianjin Hao Yang Biotech, Tianjin, China) at 1030g for 21 min at room temperature and washed twice with phosphate-buffered saline (PBS). The peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation, and peripheral blood samples were obtained from the patients immediately before thyroidectomy.

Gene expression microassay

Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology). Total RNA from thyroid and blood Tregs was linearly amplified and labeled with Cy3-UTP. The labeled cRNAs were purified using the RNeasy Mini Kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/ μ g cRNA) were measured using NanoDrop ND-1000. The following procedures were performed: 1 μ g of each labeled cRNA was fragmented by adding 11 μ L 10 $\times$ Blocking Agent and 2.2 μ L of 25 $\times$ Fragmentation Buffer, heated at 60°C for 30 min, and finally, 55 μ L 2 $\times$ GE Hybridization Buffer was added to dilute the labeled cRNA; 100 μ L of the hybridization solution was dispensed into the gasket slide and assembled on the gene expression microarray slide. The slides were incubated for 17 h at 65°C in an Agilent Hybridization Oven. The hybridized arrays were washed, fixed, and scanned using the Agilent DNA Microarray Scanner (part number G2505C). Array images were analyzed using Agilent Feature Extraction software.

Flow cytometry analysis and Treg cells isolation

Surface markers were analyzed by four-color flow cytometry with a combination of monoclonal antibodies. These antibodies included PerCP-Cy5.5-anti-CD4 (eBioscience), APC-anti-CD25 (eBioscience), FITC-anti-IFN- γ (Beckman Coulter), PE-anti-IL-4 (Beckman Coulter), APC-anti-Foxp3 (eBioscience), FITC-anti-TG (eBioscience), and PE-anti-TGF- β (eBioscience). Aliquots (100 μ L) of mononuclear cells were incubated with 10 μ L of appropriate antibody for 30 min at 4°C. About 30,000–30,500 mononuclear cells in blood and 11,000–38,000 mononuclear cells in thyroid that were dependent on size thyroid tissue pieces were labeled. For staining of anti-Foxp3, after incubation with 1 mL of fixation buffer (Becton Dickinson) for 30 min at 4°C in the dark and washing with 1 mL of permeabilization buffer (Becton Dickinson) twice, 5 μ L of anti-Foxp3 was added and incubated for 30 min at 4°C, and washed with 1 mL of permeabilization buffer twice. For staining intracellular cytokines, such as anti-IFN- γ and anti-IL-4, after stimulation for 5 h at 37°C in the dark, the cells were incubated with 10 μ L of appropriate antibody for 15 min at room temperature, and 100 μ L fixation agent (Beckman Coulter) was added to the cells. After incubation for 15 min at room temperature in the dark, the cells were washed twice and incubated with 100 μ L of permeability agent (Beckman Coulter), accompanied with anti-IFN- γ and anti-IL-4 in the dark. After incubation for 30 min, the cells were washed twice, resuspended in 500 μ L of PBS and analyzed using FACSCalibur™ flow cytometer (Becton Dickinson). The lymphocytes were analyzed by gating a lymphocyte area in a dotplot of linear forward light scatter vs linear side angle light scatter.

Treg cells and RNA isolation and quantitative real-time PCR

Real-time quantitative PCR was carried out in thyroid Tregs and peripheral blood Tregs (Control). Every group included 6–8 samples. The mononuclear cells of thyroid tissue and peripheral blood were labeled with FITC-anti-CD4 and APC-anti-CD25, and CD4+CD25+ cells were sorted using flow cytometry (BD FACSJazz™, Becton Dickinson). The cell purity was always greater than 90%, as assessed by flow cytometry analysis. After Tregs isolation, the total RNA was extracted from the blood and thyroid Tregs using TRIzol reagent (Ambion®, Life Technologies) immediately without freezing and fixing. The process of RNA extracting was followed the manufacturer's instruction of TRIzol. The RNA quantity and quality were measured by NanoDrop ND-1000. The mRNA expression was quantified by real-time PCR using ABI Prism 7500 Sequence Detector (Applied Biosystems, Life Technologies). RT-PCR were performed (SYBR® PrimeScript® RT-PCR Kit; TaKaRa Bio, Inc., Otsu, Shiga, Japan) using primers listed in Table 2. The TG (AITD3, TGN), NIS (SLC5A5, TDH1), TPO (MSA, TDH2A, TPX), FOXP3 (AIID, DIETER, IPEX, JM2, PIDX, XPID), IL-10 (CSIE, GVHDS, TGIF), TGF-β (CED, DPD1, IBDIMDE, LAP, TGFB1, IL10), IL-4 (BCGF-1, BCGF1 BSF-1, BSF1, IL4), IFN-γ (IFNG IFG, IFI) mRNAs were tested. The total reaction volume was 20 μL, and the PCR was programmed as an initial incubation for 30s at 95°C followed by 40 thermal cycles of 5s at 95°C and 34s at 60°C. In order to analyze relative gene expression with real-time PCR, we selected two reference genes, β-actin and GAPDH. The intra- and inter-assay variation was tested. The real-time PCR efficiency of the two reference genes was similar and expressions of the two reference genes were constant. We selected β-actin as reference gene and expression of the target gene was normalized to β-actin and calculated using the comparative quantification method ($2^{-\Delta\Delta C_t}$) as described previously (Yan *et al.* 2018). The relative quantity of mRNA was calculated by using equation $2^{-\Delta\Delta C_t}$. All reactions were confirmed by at least one additional independent run.

Functional analysis of Treg cells

CD4+CD25-T cells will be mixed with different proportions of CD4+CD25+ Treg cells in the presence of anti-CD3 and anti-CD28 MABs (monoclonal antibodies). The cells will be cultured for 72h in complete RPMI-1640 medium in 24-well plates. 5-(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (Sigma-Aldrich) will be added for cell labeling 15 min before cell proliferation analysis by flow cytometry. APC-anti-CD25 antibody was added for sorting CD4+CD25-T cells (T conventional cells (Tconvs)). Tregs function was analyzed using the inhibition rate of Tconvs proliferation, according to the following formula: inhibition rate (%) = proliferation rate of Tconvs alone minus proliferation rate of Tconvs co-cultured with CD4+CD25+T cells/proliferation rate of Tconvs alone × 100%.

Immunofluorescence staining

Sections from the thyroid tissue were incubated with monoclonal rabbit anti-FOXP3 (dilution 1:500) (Cell Signaling Technology) and monoclonal mouse anti-TG (dilution 1:500) (R&D Systems), which was followed by incubation with goat anti-rabbit immunoglobulin antibodies conjugated to Alexa-Fluor 488 (Dako) and for 30 min. DAPI (4',6-diamino-2-phenylindole, KEYGEN, Jiangsu, China) was used to label nuclear DNA. Appropriate isotype antibodies were used as negative controls. The labeled sections were photographed using fluorescence microscopy.

Statistical analysis

All of the experiments were run in triplicate. The statistical analysis was performed using SPSS 16.0 Software (SPSS, Inc.). The data are expressed as mean ± s.d. values. The differences between the two groups were analyzed using two-tailed Student's *t*-test. A *P* < 0.05 was considered to be statistically significant.

Table 2 Primers for real-time quantitative PCR.

Gene name	Forward primer	Reverse primer	Tm (°C)
FOXP3	TTCGAAGAGCCAGAGGACTT	GCTGCTCCAGAGACTGTACC	60°C
TG	AGGGAGAGTTTATGCCTGTCC	CAATACCCAGATACCTCAGGGAA	60°C
TPO	CTGTCACGCTGGTTATGGC	GCTAGAGACACGAGACTCCTCA	60°C
NIS	GCAGTACATTGTAGCCACGAT	TGCAGATAATTCCGGTGGACA	60°C
IL-10	GCTGGAGGACTTTAAGGGTTAC	ATGTCTGGGTCTTGTTTC	60°C
TGF-β	AACGAACTGGCTGTCTGC	CCTCTGCTCATTCCGCTTAG	60°C
IL-4	CACAAGCAGCTGATCCGATTC	TCTGGTTGGCTTCCTTCACAG	60°C
IFN-γ	TGGAGACCATCAAGGAAGAC	GCGTTGGACATTCAAGTCAG	60°C
β-actin	ATCTGCTGGAAGGTGGACAGCGA	CCCAGCACAAATGAAGATCAAGAT	60°C

Figure 1

Proportion of thyroid tissue Tregs. The thyroid tissue and peripheral blood were isolated and collected from the patients who underwent thyroid surgery; the mononuclear cells were stained for CD4, CD25, FOXP3. (A) Upper panel: T cell distribution in mononuclear cells from the thyroid tissue. Lower panel: T cell distribution in mononuclear cells from the peripheral blood. Percentage of FOXP3+CD25+ T cells in the thyroid gated on CD4+ T cells and representative dot plots are shown. (B) Frequency of CD25+FOXP3+/CD4+ T cells in the thyroid tissue and the peripheral blood. Mean $\pm$ s.d. from 12 independent experiments are shown. *** $P < 0.001$; Student's t -test.

Results

Proportion of Tregs in the thyroid tissue

To evaluate the features of T lymphocyte subsets, we compared proportion of CD4⁺ and CD4⁺CD25⁺FOXP3 cells (Tregs) in thyroid with that in peripheral blood. According to multiparameter flow cytometry, the CD4⁺ cells markedly divided into two groups, CD4^{low} and CD4^{high} cells, and most Tregs were CD4^{high} in thyroid. However, CD4⁺ cells in peripheral blood seemed to be only one group (Fig. 1A). The proportion of Tregs significantly increased in thyroid compared with that in peripheral blood (14.1 vs 1.7%, $P < 0.001$) (Fig. 1A and B).

The suppressive function of Tregs in the thyroid tissue

A standard *in vitro* suppression assay was conducted in thyroid and peripheral blood Tregs. Although thyroid Tregs exhibited general suppressive properties, the suppressive function of thyroid Tregs decreased nearly 30% compared with peripheral blood Tregs ($P < 0.001$) (Fig. 2A, B and C). The result was different from that reported in an earlier study; the suppressive function of fat Tregs was consistent with lymphoid organs-derived Tregs (Feuerer *et al.* 2009). Moreover, there was no difference between thyroid and peripheral blood Tregs in FOXP3 mRNA expression ($P > 0.05$) (Fig. 2D).

Figure 2

Functional comparison of Tregs from thyroid tissue and peripheral blood. CD4⁺CD25⁺ Tregs and conventional T (Tconv) cells were isolated from the human thyroid tissue and peripheral blood; a standard *in vitro* suppression assay was performed. Blood-derived CD4⁺ effector T cells (responder cells) were incubated with various ratios of CD4⁺CD25⁺ Tregs from the thyroid tissue and peripheral blood. (A and B) The different ratios of Tregs inhibited Tconv cell proliferation. (C) Sum of the three ratios of Tregs inhibiting Tconv cells proliferation. (D) The FOXP3 mRNA expression in the thyroid and peripheral blood Tregs. Mean $\pm$ s.d. from at least six independent experiments are shown. *** $P < 0.001$; Student's t -test.

A particular gene-expression profile of Tregs in the thyroid tissue

We wondered whether the CD25⁺FOXP3⁺ cells in thyroid tissue were of characteristic of the typical Tregs phenotype. The Tregs and Tconvs were isolated from the thyroid and peripheral blood and microarray-based gene-expression profiling was performed. According to both simple comparison plots, the transcriptome of thyroid Tregs gene differed from the patterns of its peripheral blood counterparts (Fig. 3A and B). Focusing specifically on the documented Tregs signature (Herman *et al.* 2004, Fontenot *et al.* 2005, Hill *et al.* 2007), we found that the peripheral blood data showed an excellent recapitulation of its major features: as anticipated, most genes known to be upregulated in Tregs (red) descended to the right on the *P* value vs fold-change 'volcano' plot, especially FOXP3, CD25, CTLA-4, GITR, and OX40 (Fig. 3C), whereas most downregulated loci (green) dropped to the left.

Thyroid-resident CD4⁺FOXP3⁺ cells were clearly Tregs, as most of the signatures were intact, including overexpression of hallmark transcripts like those encoding FOXP3, CD25, CTLA-4, GITR, OX40 (Fig. 3D). We also found some genes were overexpressed in thyroid Tregs, which encoded molecules involved in leukocyte migration extravasation and inflammation, including CXCL10, CXCL9, interferon (IFN)- γ , IL-4, IL10, and TGF- β , and so forth, compared with blood Tregs (Fig. 3E and F). Furthermore, the real-time PCR confirmed the results

(Fig. 3H, I and J). The results suggested that thyroid Tregs were similar to other tissue-resident Tregs (Cipolletta *et al.* 2012, Burzyn *et al.* 2013a).

High-level thyroglobulin expressed in the thyroid tissue Tregs

We unexpectedly found that thyroid-resident Tregs express thyroglobulin (Tg) compared with peripheral blood Tregs according to the transcriptome data (Fig. 3E and F). To confirm the elevated expression of Tg in thyroid Tregs, real-time PCR and immunofluorescent staining were performed. What strikes us as odd was that there were more than 500 to 1000 times of Tg mRNA expression in thyroid Tregs compared with peripheral blood counterparts ($P < 0.001$) (Fig. 3G).

We used FOXP3 and Tg fluorescent antibody to stain the thyroid tissue sections and found that thyroid Tregs simultaneously expressed FOXP3 and Tg protein (Fig. 4A). We merged the left three figures and confirmed that FOXP3 and Tg were expressed in the same cells (Fig. 4A, the right). We noted that thyroid Tregs did not disperse in the thyroid, but were concentrated in some of the thyroid follicle lumens, at the side of the thyroid follicle (Fig. 4A).

A multiparameter flow cytometry showed that $4.02 \pm 0.42\%$ CD4⁺CD25⁺ cells expressed Tg in thyroid mononuclear cells; however, there was minimal Tg expression in peripheral blood CD4⁺CD25⁺ cells ($P < 0.01$)

Figure 3

A particular gene-expression profile of Tregs in the thyroid. (A-F) Analysis with Agilent Human 4x44K Gene Expression Microarrays and (G-J) real-time PCR. (A) Normalized expression values for profiles directly comparing Tregs between the thyroid and the peripheral blood and (B) for profiles directly comparing Tconvs between the thyroid and the peripheral blood. The numbers are calculated on the basis of a cut-off of two-fold from individual comparisons, and volcano plot comparing *P* values between Tregs and Tconvs signature in the peripheral blood (C) and the thyroid (D). (E) Volcano plot comparing gene expression of the peripheral blood and thyroid Tregs. (F) Fold-change to fold-change plots comparing Tregs/Tconvs expression profiles between the peripheral blood and the thyroid. Real-time PCR comparing (G) thyroglobulin (Tg), (H) IL-4, (I) IFN- γ , and (J) IL-10 mRNA expressions between the peripheral blood and thyroid Tregs. Mean $\pm$ s.d. from six independent experiments are shown. * $P < 0.05$; *** $P < 0.001$; Student's *t*-test.

Figure 4

Protein expression of Tg in thyroid Tregs. (A) Immunofluorescence microscopy of thyroid sections. DAPI was used to label nuclear DNA. Original magnification $\times 400$. (B) Upper panel: The percentage of Tg expression in CD25+ cells from the peripheral blood gated on CD4+ T cells. Lower panel: The percentage of Tg expression in CD25+ T cells from the thyroid tissue gated on CD4+ T cells. The representative dot plots are shown. (C) The proportion of Tg+ in CD4+CD25+ cells. ** $P < 0.01$; Student's *t*-test. (D) Thyroid Tregs were isolated and various concentrations of Tregs were cultured; after 6 h, the supernatants were collected and Tg concentration was tested. Mean $\pm$ s.d. from at least five independent experiments are shown. * $P < 0.05$; ** $P < 0.01$; Student's *t*-test.

(Fig. 4B and C). Furthermore, we found that similar number of CD4⁺CD25⁻ also expressed Tg in thyroid, but this phenomenon was absent in peripheral blood (Fig. 4B). As we know, Tg, the major protein product of the thyroid gland, is synthesized by thyrocytes and is stored in the thyroid follicles (Berndorfer *et al.* 1996, Lisi *et al.* 2003). It serves as the macromolecular backbone for thyroid hormone (TH) biosynthesis by covalently incorporating iodide to its tyrosyl residues. We aimed to determine how thyroid Tregs expressed Tg and what were their roles with regard to the thyroid.

To determine whether Tg gets released freely from thyroid Tregs, we isolated thyroid Tregs with immunomagnetic beads and cultured the cells in different concentrations for 6 h. We separated the supernatant and tested the Tg using a chemiluminescence immunoassay. The results showed that Tg concentration of the supernatant increased along with the elevation of thyroid Tregs concentration (Fig. 4D).

Tg stimulates the peripheral blood Tregs to express Tg

Tg synthesis in thyroid follicular cells requires the presence of TSH (Santisteban *et al.* 1987). We wanted to know whether thyroid Tregs expression of Tg required TSH stimulation. We found that thyroid and peripheral blood Tregs all expressed TSH receptor and that thyroid Tregs expressed high levels of TSH receptor mRNA compared with peripheral blood Tregs (Fig. 5A). As peripheral blood Tregs expressed minimal amount of Tg, we stimulated Tregs of peripheral blood with 1.0 mU/mL TSH, and we found no differences in Tg mRNA expression compared with the control (Fig. 5B). However, 1.0 mg/mL Tg could stimulate peripheral blood Tregs to express Tg mRNA (Fig. 5B).

To evaluate the mechanism of Tg to stimulate Tg expression in Tregs, we used various concentrations of Tg to stimulate peripheral blood Tregs (Fig. 5C). The results suggested that a low concentration of Tg (1.0 mg/mL) could

Figure 5

Tg stimulates peripheral blood Tregs to express Tg. T-Treg represents thyroid Tregs, B-Treg represents peripheral blood Tregs. The B-Treg and T-Treg were isolated from normal human peripheral blood and thyroid, respectively. (A) TSH mRNA expression in peripheral blood and thyroid Tregs. (B) 1.0 mU/mL TSH or 1.0 mg/mL Tg was used to stimulate B-Treg. (C) The various concentrations of Tg were used to stimulate B-Treg. The mean $\pm$ s.d. from at least five independent experiments are shown. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Student's *t*-test. (D) Tg protein expression in peripheral blood CD25+ cells gated on CD4+ cells after 1.0 mg/mL Tg stimulation.

more effectively stimulate the peripheral blood Tregs to express Tg mRNA than a high level of Tg (100 mg/mL) (Fig. 5C). To confirm the Tg-stimulating Tg protein expression, we used 1.0 mg/mL of Tg to stimulate the peripheral blood mononuclear cells (PBMCs), employing multiparameter flow cytometry. After Tg stimulation, approximately $2.7 \pm 0.27\%$ of CD4⁺CD25⁺ cells expressed Tg that were significantly higher than the control (Fig. 5D).

Thyroid Tregs regulate thyroid-related gene expression in the thyroid follicular cells

The thyroid follicle is the most unique structure of the thyroid gland, where iodine is trapped, and Tg and THs are formed and stored. TSH regulates the synthesis and secretion of THs and also stimulates the synthesis of Tg, thyroid peroxidase (TPO), and the sodium/iodide symporter (Slc5a5, NIS) in order to iodinate Tg to form THs.

In addition to this well-recognized function, Tg stored in the follicle has been shown to have an unexpected role as a negative-feedback regulator of THs synthesis (Suzuki *et al.* 2011, Sue *et al.* 2012) and Tg-mediated suppression of thyroid-specific gene expression is dose dependent (Suzuki *et al.* 1998, 1999). To confirm human Tg-regulating human thyroid-related gene expression, we used human Tg to stimulate human thyroid follicular cells. The results showed that human Tg could negatively regulate Tg, TPO and NIS gene expression in a dose-dependent manner (Fig. 6A, B and C).

As thyroid Tregs expressed a high level of Tg, we wanted to determine whether thyroid Tregs also regulated thyroid function. When we co-cultured human thyroid Tregs with thyroid follicular cells, thyroid Tregs could

suppress Tg, TPO, and NIS mRNA expression, such as Tg (Fig. 6A, B and C).

Discussion

To the best of our knowledge, this study is the first description of a distinct population of Tregs in the human thyroid that expressed high levels of Tg. The exogenous Tg could induce peripheral blood CD4⁺CD25⁺ cells to express Tg mRNA, which means conventional Tregs also could transform into a particular Tregs sub-phenotype in special circumstances. It is accepted that, except for the case of the gut and placental compartment, tissue Tregs do not emanate from the conversion of conventional CD4⁺ T cells. It perhaps simply comes from FOXP3⁺, which is retained in a particular tissue and likely its TCR reacts to an antigen therein in response to tissue-specific cues, and takes on a tissue-specific sub-phenotype (Burzyn *et al.* 2013b).

The origin and differentiation of every subtype of tissue Tregs has its distinct characteristics related to the resident tissue. To some extent, the origin of thyroid Tregs is more similar to fat Tregs (Feuerer *et al.* 2009, Cipolletta *et al.* 2012), they stay in the normal tissue for relatively long term, there is no exogenous to stimulate. Tg is an endogenous antigen, Tg might interact with the TCR of Tregs therein and induce the Tregs to form the sub-phenotype or stimulate some genes of Tregs to express Tg. Whereas others may be recruited promptly when local circumstances change, such as muscle Tregs, which accumulate within days after an insult in an injured muscle (Burzyn *et al.* 2013a).

Previous studies have suggested that the thyroid follicular cells in a normal thyroid gland vary tremendously

Figure 6

Thyroid tissue Tregs affect thyroid-related gene expression. The thyroid Tregs and follicular epithelial cells were isolated from the human thyroid tissue. (A–C) The thyroid follicular epithelial cells and various concentrations of Tg or thyroid Tregs were co-cultured; the thyroid follicular epithelial cells were isolated; and real-time PCR was conducted. Mean $\pm$ s.d. from at least five independent experiments are shown, compared with the control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Student's *t*-test.

in dimension, 'active' follicles cells were tall columnar epithelium that were often in direct contact with 'inactive' follicles whose cells were in a low cuboidal to almost squamous configuration (Studer *et al.* 1978, Gerber *et al.* 1987). The persistence of follicular heterogeneity in the

mature thyroid indicates existence of some endogenous mechanisms, acting independently of classical negative endocrine feedback, which is essential for maintaining the activity of individual follicles (Yi *et al.* 1997, Sellitti & Suzuki 2014). The relatively large range of the estimates of follicular Tg concentration might be the result of significant functional heterogeneity among follicles (Sellitti & Suzuki 2014). The normal range of follicular Tg concentrations was about 0.1 mg/mL up to 250 mg/mL (Hayden *et al.* 1970, Salabe *et al.* 1996). However, how Tg concentration regulates thyroid follicular heterogeneity is not known.

The Tg not only serves as the macromolecular backbone for THs biosynthesis, but also induces growth of thyrocytes in the absence of TSH (Sue *et al.* 2012, Sellitti & Suzuki 2014) and lower concentrations of Tg (1–5 mg/mL) induced more follicular cells growth than higher concentrations (>10 mg/mL) (Suzuki & Kohn 2006, Noguchi *et al.* 2010). It has been proved that in the 'inactive' thyroid follicles, the follicular Tg concentration is maintained at a low level (Sellitti & Suzuki 2014).

In the present study, we found that a low level of Tg (1.0 mg/mL) more effectively induced peripheral blood Tregs to express Tg and Tg could be freely released from the thyroid Tregs, which were independent of TSH stimulation. The low level of Tg might stimulate the thyroid Tregs to express Tg in the 'inactive' thyroid follicles and promote follicular cell growth by Tg released from thyroid Tregs. The thyroid Tregs may be involved in the regulation of heterogeneity among the thyroid follicles by Tg, which is paracrine from the thyroid Tregs.

The human thyroid has a tendency to be attacked by autoimmune thyroid diseases (AITD), and the numerical and/or functional impairments of Tregs in peripheral blood participate in disease susceptibility and severity of AITD (Marazuela *et al.* 2006, Mao *et al.* 2011, Bossowski *et al.* 2013). However, in our study, the decline of suppressive function in thyroid Tregs seems to have no relation to AITD due to normal TPO-Ab, TG-Ab, and TR-Ab in the selected patients with benign thyroid nodule. The elevated proportion of Tregs and the high levels of IL-10 and TGF- β expression in the thyroid Tregs might be compensated for the suppressive function reduction of thyroid Tregs. The IL-10 and TGF- β also play important roles in Tregs-mediated regulatory activities. The mechanism of declined suppressive function of thyroid Tregs requires further investigation.

There are some limitations to consider in our study. First, CD25 is an activation marker for lymphocytes. The utility of CD25 expression as a Tregs marker is limited since

it does not discriminate between activated T effector (Teff) cells and Tregs. So we used FOXP3 to label the Tregs, like in flow cytometry analysis (Fig. 1) and immunofluorescence microscopy (Fig. 4). The permeabilization procedure is required in FOXP3 labeling, so we use CD25 antibody to isolate Tregs that was referenced some of the recent studies to isolate Tregs (Feuerer *et al.* 2009, Cipolletta *et al.* 2012). Due to the limitation in fluorescence labeling, we cannot label CD4, CD25, FOXP3 and TG at the same time; flow cytometry analysis also used CD25 antibody to label the Tregs (Figs 4 and 5). Second, the regulation of Tregs in some of the thyroid genes was not confirmed by depleting Tg from the Tregs-cultivated culture media in our study. Third, we found that CD4⁺CD25⁻ also expressed Tg in thyroid, which needs to be studied further. Fourth, Tregs distribution differences around 'active' and 'inactive' may provide a better understanding of the roles of Tregs in regulating thyroid function.

In summary, we have demonstrated for the first time that thyroid Tregs express a large amount of Tg and are freely released from thyroid Tregs, which might be involved in thyroid follicular cell proliferation and the regulation of thyroid follicular heterogeneity. Furthermore, thyroid Tregs could negatively regulate thyroid-related gene expressions. The present study provides new clues in the research of thyroid physiological regulation.

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.

Funding

This work was supported by the National Natural Science Foundation of China (81570710) and Nanjing Medical Science and Technology Progressing Foundation of China (ZKX15026).

Author contribution statement

XMM conceived the idea. XMM, YH and YQZ designed the experiments as well as the analyses, and they developed the method. PJ, YH, KH, and YQP performed the experiments. HZ helped with the experiments. XMM, HZ, YQZ and YH performed the analyses and interpreted the findings. XMM and HZ wrote the manuscript with input from YQZ, YH, KH and YQP. All authors corrected, revised and approved the final version of the manuscript.

References

- Berndorfer U, Wilms H & Herzog V 1996 Multimerization of thyroglobulin (TG) during extracellular storage: isolation of highly crosslinked TG from human thyroids. *The Journal of Clinical Endocrinology and Metabolism* **81** 1918–1926. (<https://doi.org/10.1210/jcem.81.5.8626858>)
- Bossowski A, Moniuszko M, Dąbrowska M, Sawicka B, Rusak M, Jeznach M, Wójtowicz J, Bodzenta-Lukaszyk A & Bossowska A 2013 Lower proportions of CD4⁺CD25^{high} and CD4⁺FoxP3, but not CD4⁺CD25⁺CD127^{low} FoxP3⁺ T cell levels in children with autoimmune thyroid diseases. *Autoimmunity* **46** 222–230. (<https://doi.org/10.3109/08916934.2012.751981>)
- Burzyn D, Kuswanto W, Kolodin D, Shadrach JL, Cerletti M, Jang Y, Sefik E, Tan TG, Wagers AJ, Benoist C, *et al.* 2013a A special population of regulatory T cells potentiates muscle repair. *Cell* **155** 1282–1295. (<https://doi.org/10.1016/j.cell.2013.10.054>)
- Burzyn D, Benoist C & Mathis D 2013b Regulatory T cells in nonlymphoid tissues. *Nature Immunology* **14** 1007–1013. (<https://doi.org/10.1038/ni.2683>)
- Campbell DJ & Koch MA 2011 Phenotypical and functional specialization of FOXP3⁺ regulatory T cells. *Nature Reviews Immunology* **11** 119–130. (<https://doi.org/10.1038/nri2916>)
- Chen X & Oppenheim JJ 2011 Resolving the identity myth: key markers of functional CD4⁺FoxP3⁺ regulatory T cells. *International Immunopharmacology* **11** 1489–1496. (<https://doi.org/10.1016/j.intimp.2011.05.018>)
- Cipolletta D 2014 Adipose tissue-resident regulatory T cells: phenotypic specialization, functions and therapeutic potential. *Immunology* **142** 517–525. (<https://doi.org/10.1111/imm.12262>)
- Cipolletta D, Feuerer M, Li A, Kamei N, Lee J, Shoelson SE, Benoist C & Mathis D 2012 PPAR- γ is a major driver of the accumulation and phenotype of adipose tissue Treg cells. *Nature* **486** 549–553. (<https://doi.org/10.1038/nature11132>)
- Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, Lee J, Goldfine AB, Benoist C, Shoelson S, *et al.* 2009 Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nature Medicine* **15** 930–939. (<https://doi.org/10.1038/nm.2002>)
- Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG & Rudensky AY 2005 Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* **22** 329–341. (<https://doi.org/10.1016/j.immuni.2005.01.016>)
- Garcia-Lopez MA, Sancho D, Sanchez-Madrid F & Marazuela M 2001 Thyrocytes from autoimmune thyroid disorders produce the chemokines IP-10 and Mig and attract CXCR3C lymphocytes. *The Journal of Clinical Endocrinology and Metabolism* **86** 5008–5016. (<https://doi.org/10.1210/jcem.86.10.7953>)
- Gerber H, Peter HJ & Studer H 1987 Age-related failure of endocytosis may be the pathogenetic mechanism responsible for "cold" follicle formation in the aging mouse thyroid. *Endocrinology* **120** 1758–1764. (<https://doi.org/10.1210/endo-120-5-1758>)
- Hayden LJ, Shagrin JM & Young JA 1970 Micropuncture investigation of the anion content of colloid from single rat thyroid follicles. A micromethod for the simultaneous determination of iodide and chloride in nanomole quantities. *Pflugers Archiv* **321** 173–186. (<https://doi.org/10.1007/bf00586371>)
- Herman AE, Freeman GJ, Mathis D & Benoist C 2004 CD4⁺CD25⁺ regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. *The Journal of Experimental Medicine* **199** 1479–1489. (<https://doi.org/10.1084/jem.20040179>)
- Hill JA, Feuerer M, Tash K, Haxhinasto S, Perez J, Melamed R, Mathis D & Benoist C 2007 Foxp3-dependent and independent regulation of the Treg transcriptional signature. *Immunity* **27** 786–800. (<https://doi.org/10.1016/j.immuni.2007.09.010>)
- Imada M, Kurosuni M & Fujita H 1986 Three-dimensional aspects of blood vessels in thyroids from normal, low iodine diet-treated, TSH-treated, and PTU-treated rats. *Cell and Tissue Research* **245** 291–296. (<https://doi.org/10.1007/bf00213934>)
- Josefowicz SZ, Lu LF & Rudensky AY 2012 Regulatory T cells: mechanisms of differentiation and function. *Annual Review of Immunology* **30** 531–564. (<https://doi.org/10.1146/annurev.immunol.25.022106.141623>)

- Lisi S, Pinchera A, McCluskey RT, Willnow TE, Refetoff S, Marcocci C, Vitti P, Menconi F, Grasso L, Luchetti F, *et al.* 2003 Preferential megalin-mediated transcytosis of low-hormonogenic thyroglobulin: a control mechanism for thyroid hormone release. *Proceedings of the National Academy of Sciences of the United States of America* **100** 14858–14863. (<https://doi.org/10.1073/pnas.2432267100>)
- Lu LF & Rudensky A 2009 Molecular orchestration of differentiation and function of regulatory T cells. *Genes & Development* **23** 1270–1282. (<https://doi.org/10.1101/gad.1791009>)
- Mao C, Wang S, Xiao Y, Xu J, Jiang Q, Jin M, Jiang X, Guo H, Ning G & Zhang Y 2011 Impairment of regulatory capacity of CD4+CD25+ regulatory T cells mediated by dendritic cell polarization and hyperthyroidism in Graves' disease. *Journal of Immunology* **186** 4734–4743. (<https://doi.org/10.4049/jimmunol.0904135>)
- Marazuela M, García-López MA, Figueroa-Vega N, de la Fuente H, Alvarado-Sánchez B, Monsiváis-Urenda A, Sánchez-Madrid F & González-Amaro R 2006 Regulatory T cells in human autoimmune thyroid disease. *The Journal of Clinical Endocrinology and Metabolism* **91** 3639–3646. (<https://doi.org/10.1210/jc.2005-2337>)
- Marazuela M, Postigo AA, Acevedo A, Díaz-González F, Sanchez-Madrid F & de Landázuri MO 1994 Adhesion molecules from the LFA-1/ICAM-1,3 and VLA-4/VCAM-1 pathways on T lymphocytes and vascular endothelium in Graves' and Hashimoto's thyroid glands. *European Journal of Immunology* **24** 2483–2490. (<https://doi.org/10.1002/eji.1830241034>)
- Noguchi Y, Harii N, Giuliani C, Tatsuno I, Suzuki K & Kohn LD 2010 Thyroglobulin (Tg) induces thyroid cell growth in a concentration-specific manner by a mechanism other than thyrotropin/cAMP stimulation. *Biochemical and Biophysical Research Communications* **391** 890–894. (<https://doi.org/10.1016/j.bbrc.2009.11.158>)
- Oda K, Luo Y, Yoshihara A, Ishido Y, Sekihata K, Usukura K, Sue M, Hiroi N, Hirose T & Suzuki K 2017 Follicular thyroglobulin induces cathepsin H expression and activity in thyrocytes. *Biochemical and Biophysical Research Communications* **483** 541–546. (<https://doi.org/10.1016/j.bbrc.2016.12.109>)
- Ohkura N, Hamaguchi M & Sakaguchi S 2011 FOXP3+ regulatory T cells: control of FOXP3 expression by pharmacological agents. *Trends in Pharmacological Sciences* **32** 158–166. (<https://doi.org/10.1016/j.tips.2010.12.004>)
- Richards DM, Delacher M, Goldfarb Y, Kägebein D, Hofer AC, Abramson J & Feuerer M 2015 Treg cell differentiation: from thymus to peripheral tissue. *Progress in Molecular Biology and Translational Science* **136** 175–205. (<https://doi.org/10.1016/bs.pmbts.2015.07.014>)
- Salabe GB, Corvo L & Lotz H 1996 Thyroglobulin determined in thyroid fine needle aspiration biopsies by radial immunodiffusion and electroimmunodiffusion. *European Journal of Clinical Chemistry and Clinical Biochemistry* **34** 43–47. (<https://doi.org/10.1515/cclm.1996.34.1.43>)
- Santisteban P, Kohn LD & Di Lauro R 1987 Thyroglobulin gene expression is regulated by insulin and insulin-like growth factor I, as well as thyrotropin, in FRTL-5 thyroid cells. *Journal of Biological Chemistry* **262** 4048–4052.
- Sellitti DF & Suzuki K 2014 Intrinsic regulation of thyroid function by thyroglobulin. *Thyroid* **24** 625–638. (<https://doi.org/10.1089/thy.2013.0344>)
- Shevach EM 2009 Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* **30** 636–645. (<https://doi.org/10.1016/j.immuni.2009.04.010>)
- Studer H, Forster R, Conti A, Kohler H, Haeberli A & Engler H 1978 Transformation of normal follicles into thyrotropin-refractory “cold” follicles in the aging mouse thyroid gland. *Endocrinology* **102** 1576–1586. (<https://doi.org/10.1210/endo-102-5-1576>)
- Sue M, Hayashi M, Kawashima A, Akama T, Tanigawa K, Yoshihara A, Hara T, Ishido Y, Ito T, Takahashi S, *et al.* 2012 Thyroglobulin (Tg) activates MAPK pathway to induce thyroid cell growth in the absence of TSH, insulin and serum. *Biochemical and Biophysical Research Communications* **420** 611–615. (<https://doi.org/10.1016/j.bbrc.2012.03.046>)
- Suzuki K, Kawashima A, Yoshihara A, Akama T, Sue M, Yoshida A & Kimura HJ 2011 Role of thyroglobulin on negative feedback autoregulation of thyroid follicular function and growth. *The Journal of Endocrinology* **209** 169–174. (<https://doi.org/10.1530/JOE-10-0486>)
- Suzuki K & Kohn LD 2006 Differential regulation of apical and basal iodide transporters in the thyroid by thyroglobulin. *The Journal of Endocrinology* **189** 247–255. (<https://doi.org/10.1677/joe.1.06677>)
- Suzuki K, Lavaroni S, Mori A, Ohta M, Saito J, Pietrarelli M, Singer DS, Kimura S, Katoh R, Kawaoi A, *et al.* 1998 Autoregulation of thyroid-specific gene transcription by thyroglobulin. *Proceedings of the National Academy of Sciences of the United States of America* **95** 8251–8256. (<https://doi.org/10.1073/pnas.95.14.8251>)
- Suzuki K, Mori A, Saito J, Moriyama E, Ullianich L & Kohn LD 1999 Follicular thyroglobulin suppresses iodide uptake by suppressing expression of the sodium/iodide symporter gene. *Endocrinology* **140** 5422–5430. (<https://doi.org/10.1210/endo.140.11.7124>)
- Yan Z, Dai Y, Fu H, Zheng Y, Bao D, Yin Y, Chen Q, Nie X, Hao Q, Hou D, *et al.* 2018 Curcumin exerts a protective effect against premature ovarian failure in mice. *Journal of Molecular Endocrinology* **60** 261–271. (<https://doi.org/10.1530/JME-17-0214>)
- Yi X, Yamamoto K, Shu L, Katoh R & Kawaoi A 1997 Effects of propylthiouracil (PTU) administration on the synthesis and secretion of thyroglobulin in the rat thyroid gland: a quantitative immunoelectron microscopic study using immunogold technique. *Endocrine Pathology* **8** 315–325. (<https://doi.org/10.1007/BF02739934>)

Received in final form 11 September 2019

Accepted 16 September 2019

Accepted Preprint published online 16 September 2019