Original Paper

Imbalance of Th17/Treg in Different Subtypes of Autoimmune Thyroid Diseases

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Key Words

Graves' disease (GD) • Hashimoto's thyroiditis (HT) • Graves' ophthalmopathy (GO) • T helper 17 cell (Th17) • Regulatory T cell (Treg)

Abstract

Aims: To clarify the imbalance of Th17/Treg in different subtypes of autoimmune thyroid diseases (AITDs) including Graves' disease (GD), Hashimoto's thyroiditis (HT) and Graves' ophthalmopathy (GO). **Methods:** 47 patients with AITD (including 16 GD, 15 HT, and 16 GO) and 12 healthy controls were enrolled in this study. The percentages of Th17 and Treg cells, the ratio of Th17/Treg, as well as their related transcription factors ROR γ t and Foxp3 mRNA in peripheral blood mononuclear cells (PBMCs) were measured by flow cytometry and real-time quantitative PCR. **Results:** Compared with those in control group, the percentage of CD4⁺IL-17⁺T cell (Th17) and the mRNA expression of its transcription factor ROR γ t were higher in PBMCs of AITDs ($P < 0.05$), particularly in HT subgroup ($P < 0.01$). The percentage of CD4⁺Foxp3⁺T (Treg) cells and its transcription factor Foxp3 mRNA were significantly decreased in PBMCs of GD ($P < 0.05$). In addition, the ratio of Th17/Treg was elevated in AITD group and GO subgroup ($P < 0.01$). In GO subgroup, the patients with clinical activity score (CAS) above 4.5 had higher percentages of Th17 than those with CAS ranging from 3 to 4.5 ($P < 0.05$). **Conclusion:** Increased Th17 lymphocytes may play a more important role in the pathogenesis of HT and GO while decreased Treg may be greatly involved in GD.

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Introduction

Autoimmune thyroid diseases (AITDs), a group of organ-specific autoimmune diseases, mainly include Graves' disease (GD), Hashimoto's thyroiditis (HT), and Graves' ophthalmopathy (GO). GO is the most frequent extrathyroidal manifestation of GD and also the most common cause of adult exophthalmos. It occurs in about 50% of patients with GD,

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and 3-5% is sight-threatening [1]. It is suggested that the environmental factors trigger the occurrence of AITDs under genetical background. However, the specific mechanism of AITDs yet remains unknown, particularly, the causes the different subtypes of AITDs with a variety of manifestations. Furthermore, we and other investigators have found that distinct AITDs phenotypes may even share common susceptibility genes [2, 3]. In our another study, we found that the miRNA-target gene network might be involved in the pathogenesis of GD [4].

Advances in cellular immunology have opened a new era of or new insights into exploring the mechanisms of immune related diseases. It is well known that Th1/Th2 cell equilibrium is necessary to maintain the normal balance of the immune system. The disruption of Th1/Th2 balance is attributed to the development of many autoimmune diseases. In HT, Th1-type cell and its cytokines-mediated cellular immune response are overwhelming [5-7]. While in GD, Th2-type cytokines-mediated humoral immune response is predominant [7, 8]. Th17 cell, a recently identified T helper lymphocyte of CD4 positive, acts by secreting its main cytokine IL-17A. It has been well documented that Th17 cell is implicated in the development of different infections, various inflammations and carcinomas [9-11]. Regulatory T cell (Treg), another type of lymphocytes, negatively regulates immune responses and confine them to a proper extent [12]. Like Th1/Th2, Th17/Treg is also a pair of balance in the immune system. It has been shown that Th17/Treg homeostasis was broken in multiple autoimmune diseases [13, 14]. We and other investigators once conducted research on Th17 or Treg lymphocytes in GD or HT [15-17]. However, there has been neither study on Th17/Treg in GO nor a comparative analysis of Th17/Treg in different types of AITDs. In the present study, we investigated Th17/Treg cells in three types of AITDs simultaneously and comparatively.

Material and Methods

Subjects: Forty-seven newly diagnosed AITDs patients (16 with GD, 16 with GO and 15 with HT) were recruited. They had never received any anti-thyroid drugs or immune regulators. Neither did they have other committant autoimmune diseases or allergic diseases. The diagnoses of HT and GD were made on the basis of clinical manifestations and laboratory results as well as the criteria in our previous paper [15]. The ocular complication of GD, i.e. GO, was diagnosed according to symptoms and signs as well as orbital imaging (CT and MRI). Twelve healthy volunteers were selected as controls. The demographics and clinical characteristics of the subjects are shown in Table 1. The clinical activity score (CAS) of each GO patient was determined according to the standard of American Thyroid Association [18] as shown in Table 2. The research project was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University.

Methods

Blood Samples. Peripheral venous blood samples were collected from the patients and healthy volunteers. All subjects fasted for 12 h. The collection tubes contained 0.2 mL of sodium heparin. The peripheral blood mononuclear cells (PBMCs) were prepared using Ficoll-Hypaque density centrifugation and then stored at -80°C with Trizol for extracting total mRNA. Other PBMCs were separated into two tubes for flow cytometric analysis.

Flow cytometric analysis of Treg and Th17. PBMCs were separated into $2-6 \times 10^6/\text{ml}$ in each tube. For Treg assay, PBMCs were incubated with anti-human CD4-FITC (BD Bioscience, USA) at 4°C for 30 min in darkness. After fixation and permeabilization, cells were stained with anti-human Foxp3-PE (BD Bioscience, USA) for 30 min at 4°C in darkness. As for Th17 assay, cells were stimulated with phorbol myristate acetate (PMA 50 ng/mL) and ionomycin (1 $\mu\text{g}/\text{mL}$) (America Enzo life) with the presence of monensin (0.7 $\mu\text{L}/\text{mL}$) (BD Bioscience, USA) at 37°C and 5% CO_2 for 4 h. Then they were stained with anti-human CD4-FITC. Fixation and permeabilization were performed with fix/perm buffer. Cell pellet was resuspended in 100 μL BD Perm/Wash™ buffer (BD Bioscience, USA). For the staining of Th17 cells internal antigen, anti-human IL-17-PE was added at the bottom of the tube, vortexed and incubated for 30 min at 4°C in the dark. Cells were washed with perm/wash buffer (1 $\times$)(2ml), and then were centrifuged 5 min at 500g at room temperature.

Table 1. Demographics and clinical characteristics of patients and controls. Data are shown as mean±SD. Con: control; M: male; F: female; FT3: free T3; FT4: free T4; TgAb: thyroglobulin antibody; TPOAb: thyroperoxidase antibody

Groups	GD	GO	HT	Con
Number (M/F)	16(4/12)	16(3/13)	15(3/12)	12(3/9)
Age (yr)	39.25±11.84	39.60±6.57	38.00±10.98	36.88±6.29
T3(μg/dL)	10.66±18.73	2.11±1.25	1.67±0.93	11.17±1.4
T4 (μg/dL)	29.41±30.45	21.19±49.89	14.47±22.30	6.58±1.58
FT3(ng/mL)	32.06±16.74	11.31±9.36	7.87±6.71	5.53±1.92
FT4 (ng/mL)	91.39±54.19	27.18±23.93	14.6 ±3.94	6.90±2.60
TSH (μU/mL)	0.08±0.06	1.48±2.08	4.69±2.94	2.78±0.42
TgAb (%)	28.41±19.38	25.39±18.99	215.29±656.27	<30%
TPOAb (U/mL)	1607.20±1494.32	1379.9±1412.99	2070.51±1345.23	<15

Table 2. Clinical features and CAS of patients with GO. Patient number: 16. M=male, F=female; CAS: Clinical activity Score. The activity score range from 3 to 9, which indicated active Graves' ophthalmopathy

Patient number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Sex	F	M	F	F	F	F	F	F	F	M	F	F	M	F	F	F
Age	30	35	48	38	44	25	40	46	49	35	36	41	39	44	38	46
Pain	+		+	+	+	+	+	+		+	+	+	+	+		+
Redness of eyelids	+	+	+	+	+	+		+	+	+	+		+	+		+
Redness of conjunctiva	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chemosis	+	+	+	+			+	+	+	+	+	+	+		+	+
Swollen caruncula			+			+				+			+			
Eyelid edema			+			+				+			+			+
Increased proptosis					+	+				+	+	+	+			
Decrease in visual acuity					+	+				+	+	+			+	+
Decrease in motility										+	+	+				
Clinical activity score (CAS)	4	3	6	4	5	7	3	4	3	9	7	6	7	3	3	6

In the end, the internal staining of IL-17 was analyzed immediately by flow cytometry using a BD FACS Calibur (BD Pharmingen), or suspended with 2% formaldehyde solution of PBS until they were analyzed during 24 hours.

Real time quantitative PCR

Total mRNA from stored PBMCs was extracted with the Trizol reagent (TianGen Biotech) according the manufacturer's instructions. Then the mRNA was reversely transcribed to cDNA with reverse transcription reagent kits (TianGen Biotech). The reverse transcription system consists of random 6 mers and primerscript RT enzyme mix 1 (Primescript RT reagent kit, TakaRa, China). The PCR amplification of cDNA was performed using SYBR Premix EX Taq II (Primescript RT reagent kit). The sequences of primers were as follows: for RORγt, sense, 5'-CTG CCC ATC ATT GCT GTT AAT CC-3'; antisense, 5'-GCT GTG ATC TTG CCC AGA ACC-3'; for Foxp3, sense, 5'-CTA CGC CAC GCT CAT CCG CTG G-3'; antisense, 5'-GTA GGG TTG GAA CAC CTG CTG GG-3'; for β-actin, sense, 5'-ATC GTG CGT GAC ATT AAG GAG AAG-3'; antisense, 5'-AGG

Fig. 1. The expression of CD4⁺IL-17⁺ lymphocytes and CD4⁺Foxp3⁺ Tregs in PBMCs from patients with GD, HT, GO and healthy controls by flow cytometry.

AAG AAG GCT GGA AGA GTG-3'. Gene specificity was confirmed by a single peak in melting curve analysis. Amplification cycles (40 cycles) were as follows: 3 min at 95 °C, 5 sec at 95 °C, 30 sec at 61 °C, 45 sec at 72 °C.

Statistical analysis

All continuous data are expressed as mean ± SD. Data were analyzed using parametric and nonparametric tests. Mean group values were compared by ANOVA. Paired t test and Wilcoxon rank-sum test were used for related two samples. The data were processed using chi-square test. For the data of RT-PCR, we used the $2^{-\Delta\Delta CT}$ method. $P < 0.05$ was considered as statistically significant. SPSS 13.0 was used for statistical analysis.

Results

In our study, we measured the percentages of Th17 and Treg cells by flow cytometry (Fig. 1). It showed that the percentage of CD4⁺IL-17⁺T cells was significantly higher in patients with HT ($P = 0.003$), GD and GO ($*P < 0.05$) than in normal controls (Fig. 2A). On the other hand, the percentage of CD4⁺Foxp3⁺Treg cells was lower in the patients with AITDs, especially GD ($P = 0.047$), although there was no significant difference between HT and GD (Fig. 2B). Also the ratio of Th17/Treg was higher ($**P < 0.01$) in the patients with AITDs than in controls (Fig. 2C). In contrast, in patients with GO, the percentage of Th17 was significantly increased ($P = 0.035$) in the ones with CAS above 4.5 when compared with those with CAS between 3 and 4.5 (Fig. 2D). Regrettably, we did not find the correlation between the Th17/Treg ratio and GO activity.

When we detected the expression of different transcription factors RORγt and Foxp3 mRNA by real-time PCR, we found the levels of RORγt mRNA were significantly augmented in patients with AITDs compared with those in normal controls. These results were much in agreement with the above cellular analyses. Additionally, RORγt mRNA levels were even much higher in HT than in GD and GO ($*P < 0.05$) patients (Fig. 3A).

In comparison with that in healthy controls, the transcriptional level of Foxp3 was obviously decreased in GD and GO ($*P < 0.05$) patients (Fig. 3B). Although the expression of Foxp3 mRNA was also reduced in HT patients, the difference was not significant.

Fig. 2. The frequency of CD4⁺IL-17⁺T cells and CD4⁺Foxp3⁺Tregs in AITD patients and controls. The percentages of Th17 lymphocytes and Tregs were detected by flow cytometry. Black bars correspond to HT, dark gray bars to GD, gray bars to GO, White bars to Con (A, B, C). *, P<0.05; **, P<0.01. Con, control.

Fig. 3. The expression of RORγt and Foxp3 mRNAs in AITDs patients and normal controls. A. The expression of orphan retinoic acid nuclear receptor(RORγt) is increased in AITDs than in controls(*, P<0.05; **, P<0.01). Contrast to GD and GO, the expression of RORγt is much higher in HT (*, P<0.05). B. The expression of mRNA levels of forkhead box P3(Foxp3) is decreased in GD and GO than in healthy control(*, P<0.05).

Discussion

Th17 lymphocyte is a newly discovered T helper cell characterized by the production of IL-17A. Treg, a kind of immune regulating lymphocyte, plays a negative role by secreting cytokines like IL-10 and TGF-β. It has been reported that Th17/Treg imbalance is involved in the development of multiple immune related diseases.

In our study, we found an apparently reduced percentage of CD4⁺Foxp3⁺Treg in GD patients (Fig. 2B), together with a decreased expression of its transcription factor Foxp3(Fig.

3B). Similar to our results, the frequency of CD4⁺CD25⁺Foxp3⁺T cells and the expression of Foxp3 mRNA were found decreased significantly in the peripheral blood cells of Ad-TSH289 immunized mice, an animal model of GD. But there was no difference in Th17 cells from those in Ad-control mice (19). It probably indicates that Tregs, but not Th17 cells, plays a predominant role in the pathogenesis of GD. Our findings are also consistent with Klatka's. They found lower percentages and absolute counts of Treg cells in GD teenagers as compared with healthy adolescents [17]. A little different from us, in Nanba's study, they found the percentage of peripheral Th17 cells was higher in patients with intractable GD, whose anti-thyrotropin receptor antibody (TRAb) remained positive despite treatment with anti-thyroid drugs for more than 5 years [20].

Other researchers have observed an increase in the number of Th17 cells in the spleen and thyroid tissue of iodine-induced autoimmune thyroiditis in nonobese diabetic-H2h4 mice, a mouse model of HT. However, the severity of intrathyroidal lymphocyte infiltration, also the titers of antithyroglobulin autoantibodies, was obviously reduced in iodine-treated IL-17^{-/-} mice when compared with wild-type mice, so they believed that Th17 exerts an important role in the pathogenesis of autoimmune thyroiditis [21]. Nicte and his colleagues explored Th17 lymphocytes and its cytokines (IL-17, IL-22, IL-6 and IL-23) in the peripheral blood of AITD patients and the results showed the number of Th17 lymphocyte and the synthesis of its cytokines were enhanced, especially in HT [22]. Also another study showed that intrathyroid infiltrating Th17 cells and serum IL-17 levels were significantly increased in HT patients [16]. In our work, Th17 lymphocyte (Fig. 2A) and its transcription factor RORγt mRNA (Fig. 3A) increased in patients with AITDs and RORγt mRNA increased significantly in patients with HT compared with those with GD. Therefore, we speculated that Th17 has a critical role in the pathogenesis of HT.

For a long time, people have been interested in the mechanism of GO. Not only the number of CD4⁺T and the ratio of CD4⁺/CD8⁺T are considerably increased in GO patients, but also the percentage of Th1 lymphocytes and the ratio of Th1/Th2 cells are significant higher in patients with GO, indicating that Th1-type CD4⁺ cell might play a dominant role in the pathogenesis of GO [23]. Other researchers analyzed T helper (Th) cell subsets in orbital adipose/connective tissues of GO patients, and Th1-type clone was detected predominantly in patients with recent onset (<2 yr). In contrast, Th2-type clone was overwhelming in patients with more remote onset (>2 yr) of the disease [24]. Our team found an increased number of CD4⁺IL-17⁺T cells and a higher expression of RORγt mRNA in GO patients, but not in GD patients. Furthermore, the peripheral Th17 cells significantly enhanced in patients with severe GO (CAS>4.5) than those with mild disease (CAS between 3 and 4.5)(Fig. 2D). Interestingly, unlike GD, the difference in Tregs and its transcription factor (Foxp3) was found not to be significant between GO patients and healthy controls. Therefore, we speculated that Th17 had a critical influence on the development of GO, while Tregs mainly participated in the pathogenesis of GD.

To conclude, our research demonstrated the breaking of Th17/Treg balance in AITDs. We here initially reported that the immune response shift to Th17 was more pronounced in GO than in HT and GD. In addition, this immune bias was associated with the disease activity of GO. Our study may have practical applications. For example, it has already documented that IL-17A neutralization may alleviate the early stage of silica-induced lung inflammation and delay its progression [25]. Quite recently, a bispecific anti-TNFα/IL-17 antibody showed superior efficacy in blocking inflammatory cytokine and chemokine responses in the culture of human fibroblast-like synoviocytes from rheumatoid arthritis, and that this antibody displayed a protective impact on the bone of arthritic mice [26]. Therapeutic approaches for AITDs depending on correcting the imbalance of Th17/Treg are expected.

Funding

This work was supported by grants from the National Natural Science Foundation of China (81070627, 81270871).

Acknowledgements

The authors thank all of the participants in the present study including members of the Medical Laboratory Center of Xi'an Jiaotong University.

Disclosure Statement

The authors declare no financial or commercial conflict of interest.

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