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Original Article

Increased frequencies of nuocytes in peripheral blood from patients with Graves' hyperthyroidism

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Abstract: Newly identified nuocytes play an important role in Th2 cell mediated immunity such as protective immune responses to helminth parasites, allergic asthma and chronic rhinosinusitis. However, the contributions of nuocytes in the occurrence and development of Graves' hyperthyroidism remains unknown. Previous studies found that there was a predominant Th2 phenotype in patients with Graves' hyperthyroidism, it might relate to polarization of nuocytes. Nuocytes were defined by transcription factor *RORα*, various cell surface markers (T1/ST2, IL-17RB, ICOS, CD45) and associated cytokines. In this study, these cells related genes or molecules in PBMC from patients with Graves' hyperthyroidism were measured, and the potential correlation between them was analyzed. The expression levels of T1/ST2, IL-17RB, ICOS, IL-5 and IL-13, which represented nuocytes associated molecules were significantly increased in patients, meanwhile, the *RORα* mRNA also had a tendency to increase. In addition, IFN-γ and *T-bet* (Th1 related cytokine and transcription factor) were obviously decreased, and there was a positive correlation between IL-17RB and IL-13. These results suggested that there were polarized nuocytes in Graves' hyperthyroidism patients, and which closely related to the down-regulation of Th1 cells or relatively advantage of Th2 differentiation.

Keywords: Nuocytes, cytokines, transcription factors, Graves' hyperthyroidism

Introduction

Autoimmune thyroid diseases (AITDs), including graves' hyperthyroidism (Graves' disease, GD) and Hashimoto's thyroiditis (HT), are among the most common human autoimmune diseases. Graves' disease is an organ-specific autoimmune disease that causes hyperthyroidism via the production of TSH or thyrotropin receptor autoantibodies which stimulate the thyroid, and HT is characterized by apoptosis of thyrocytes leading to hypothyroidism and the presence of thyroid peroxidase antibodies (TPOAb) or antibodies against thyroglobulin (TGAb). Graves' disease is known to have a genetic-environmental etiology. Due to its ability to stimulate the growth of thyroid nodules, it may be accompanied by an autoimmune lymphocytic disease, and hyperplastic adenomatous tissue [1].

T helper cells are a group of immune cells that mediate adaptive immunity in vertebrates and are comprised of four major subtypes, Th1, Th2, Th17, and Treg [2-5]. In comparison to other T helper subsets, Th2 cells are present in upper level in several autoimmune diseases, in which antibodies are produced. The pathogenesis of Graves' hyperthyroidism is complex and heterogeneous, and its etiology remains unclear. Since TSAb is a hallmark of graves' hyperthyroidism, Th2 responses have been associated with the pathogenesis of graves' hyperthyroidism. Strikingly, recent studies have suggested that other types of functional T cells, such as Th17 cells, also play an important role in the pathogenesis of Graves' hyperthyroidism [6]. However, there is little information available about the role of other types of immunocompetent cells in the development and progression of Graves' hyperthyroidism, especially newly identified nuocytes, which play an important role in the promotion of Th2 cell polarization.

Nuocytes are dependent on GATA-binding protein 3 (*GATA3*) [7, 8] and retinoic acid receptor-

Table 1. The primer sequences for RT-PCR

Gene	Sequence (5'-3')	Accession
GATA3	Fwd: TTGTGGTGGTCTGACAGTTC	NM_002051
	Rev: AGTACAGCTCCGGACTCTTC	
RORα	Fwd: CTGACGAGGACAGGAGTAGG	NM_134261
	Rev: GTGCGCAGACAGAGCTATTC	
T-bet	Fwd: AATGCAGGCTTCATGCTGAC	NM_013351.1
	Rev: GCCTACCAGAATGCCGAGAT	
IL-17RB	Fwd: CTTGGTGGCCTTCAACAAGC	NM_018725
	Rev: AGAGCCGACCGTTCAATGTG	
T1/ST2	Fwd: AGATGAGTCACTGGCATACG	NM_016232
	Rev: GAGAGGCTGGCTGTTGTATT	
ICOS	Fwd: GGCATGAGAATGGTCCAAGT	NM_012092
	Rev: CATGAAGTCAGGCCTCTGGT	
CD45	Fwd: GTGAGGCGTCTGTACTGATG	NM_002838
	Rev: ACGGCTGACTTCCAGATATG	
IL-13	Fwd: GGCTGAGGTCTAAGCTAAGG	NM_002188
	Rev: GACAGCTGGCATGTACTGTG	
IL-5	Fwd: ACTCTCCAGTGTGCCTATTC	NM_000879
	Rev: CTGCTGATAGCCAATGAGAC	
IL-4	Fwd: GACATCTTTGCTGCCTCCA	NM_000589.3
	Rev: TACTCTGGTTGGCTTCCTTCA	
IFN-γ	Fwd: TTGGGTTCTCTTGGCTGTTACT	NM_000619
	Rev: ATCCGCTACATCTGAATGACCT	
β-actin	Fwd: TGGCACCCAGCACAATGAA	XM_005249820.1
	Rev: CTAAGTCATAGTCCGCCTAGAAGCA	

related orphan receptor-α (*RORα*) for their development and function [9]. Human nuocytes are, therefore, defined by (1) various cell surface markers (i.e., IL-7Rα, CD161, CRTH2, ICOS, CD45); (2) the expression of receptors for the cytokines IL-33 (T1/ST2) and IL-25 (IL-17RB); or (3) their production of the ''type 2'' cytokines IL-13, IL-5 and IL-4 [10, 11]. In a previous study, it was found that there was a predominant Th2 phenotype in patients with Graves' hyperthyroidism [12], while several researches have demonstrated that newly identified nuocytes promote and induce the development of $CD4^+$ Th2 cell-dependent immunity [13]. Thus, we proposed a hypothesis that there may be nuocytes in peripheral blood which related to Graves' hyperthyroidism. In this study, we characterized the frequency of peripheral blood nuocytes and analyzed nuocytes related factors, investigated the relationship between nuocytes and the development of Graves' hyperthyroidism, which accumulating the new information for further research immune status in patients with Graves' hyperthyroidism.

Methods

Patients and specimens

Twenty-eight patients diagnosed newly with Graves' hyperthyroidism from December 2013 to May 2014 at the Affiliated People's Hospital of Jiangsu University were included in the study, 20 females and 8 males, ranging in age 21 to 65 years (average age, 42.89 years). All the patients were untreated for their condition at the time of blood collection. The diagnosis of Graves' hyperthyroidism was based on commonly accepted clinical and laboratory criteria. 21 healthy volunteers were studied simultaneously as control, including 13 females and 8 males ranging in age from 20 to 68 years (average age, 39.08 years). This study was approved by the ethical committee of the Affiliated People's Hospital of Jiangsu University.

Peripheral blood samples were collected, plasma were frozen at -80°C immediately after centrifugation for future use. Peripheral blood mononuclear cells (PBMCs) were obtained by standard Ficoll-Hypaque density centrifugation (Tianjin Hao Yang Company, China), then with 1 ml Trizol (Invitrogen, USA) and stored at -80°C for extract total RNA.

RNA extraction and quantitative real-time PCR

Total RNA was extracted by guanidinium thiocyanate phenol chloroform method, total RNA (500 ng) was reverse transcribed using PrimeScript® RT reagent Kit Perfect Real Time (TaKaRa, Dalian, China), according to the manufacturer's instructions. On the basis of Genebank sequences, the primers used in this study were designed by Premier 5.0 software and synthesized by Shanghai Invitrogen. All sequences of primers are shown in **Table 1**. Quantitative Real-time PCR (qRT-PCR) was conducted in a SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China) according to the manufacturer's instructions. Fold changes in the expression of each objective gene relative to β-actin were calculated based on the threshold cycle (Ct). All samples were performed in triplicate.

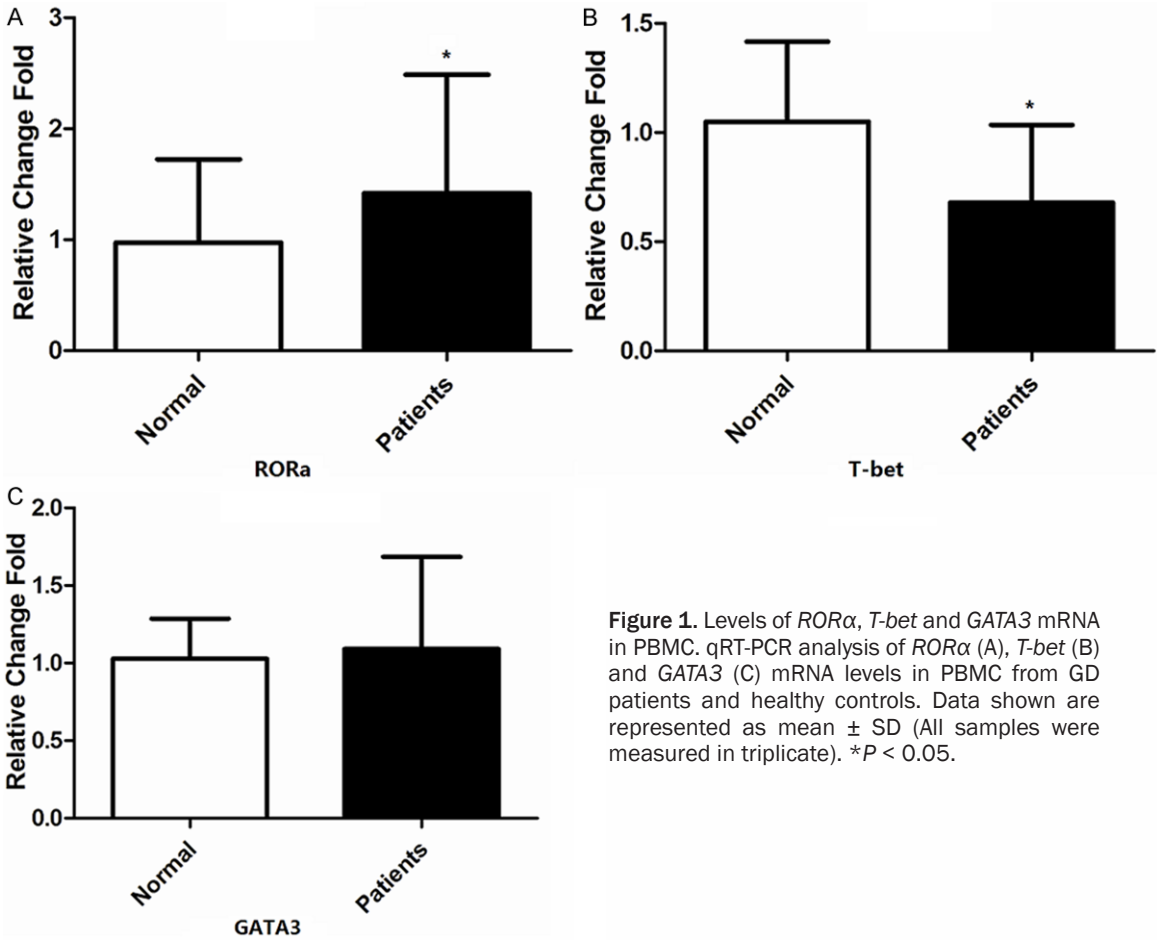

Figure 1. Levels of *RORα*, *T-bet* and *GATA3* mRNA in PBMC. qRT-PCR analysis of *RORα* (A), *T-bet* (B) and *GATA3* (C) mRNA levels in PBMC from GD patients and healthy controls. Data shown are represented as mean ± SD (All samples were measured in triplicate). $*P < 0.05$.

ELISA for plasma cytokines

Plasma levels of IL-13 (Shanghai ExCell Biology, China) and IL-5 (eBioscience, USA) were measured by ELISA kit following the manufacturer's protocols. All samples were measured in triplicate, and the mean concentration was calculated from the standard curve.

Flow cytometric quantification of nuocytes

Nuocytes population was defined as Lin^- $ICOS^+IL\text{-}17RB^+$. Heparinized venous blood was freshly obtained from GD patients or healthy volunteers. PBMCs were isolated by standard Ficoll-Hypaque density centrifugation (GE Healthcare), and stained with the following antibody mix: FITC-conjugated anti-human CD2, CD3, CD14, CD16, CD19, CD56, CD235a (eBioscience, USA); Allophycocyanin (APC)-conjugated anti-human ICOS and Peridinin chlorophyll Protein Complex (PerCP)-conjugated anti-human IL-17RB (R & D, USA). The isotype control antibody was used in all cases. For FACS phenotype analysis, data were acquired on an Accuri C6 (BD company) and analyzed with FlowJo software (TreeStar, Inc.).

Statistical analysis

All statistical analysis was performed using GraphPad Prism Version 5.0 software (San Diego, CA, United States). Data are expressed as the mean ± SD in figures. Comparisons between groups were performed using the Unpaired Student's t-test. Pearson's correlation was used to test correlation between two continuous variables. $P < 0.05$ was considered to be statistically significant.

Results

Enhanced expression levels of RORα in PBMC from patients with Graves' hyperthyroidism

The transcription factors *RORα* is essential for the development and function of human nuocytes. To analyze the level of nuocytes in the

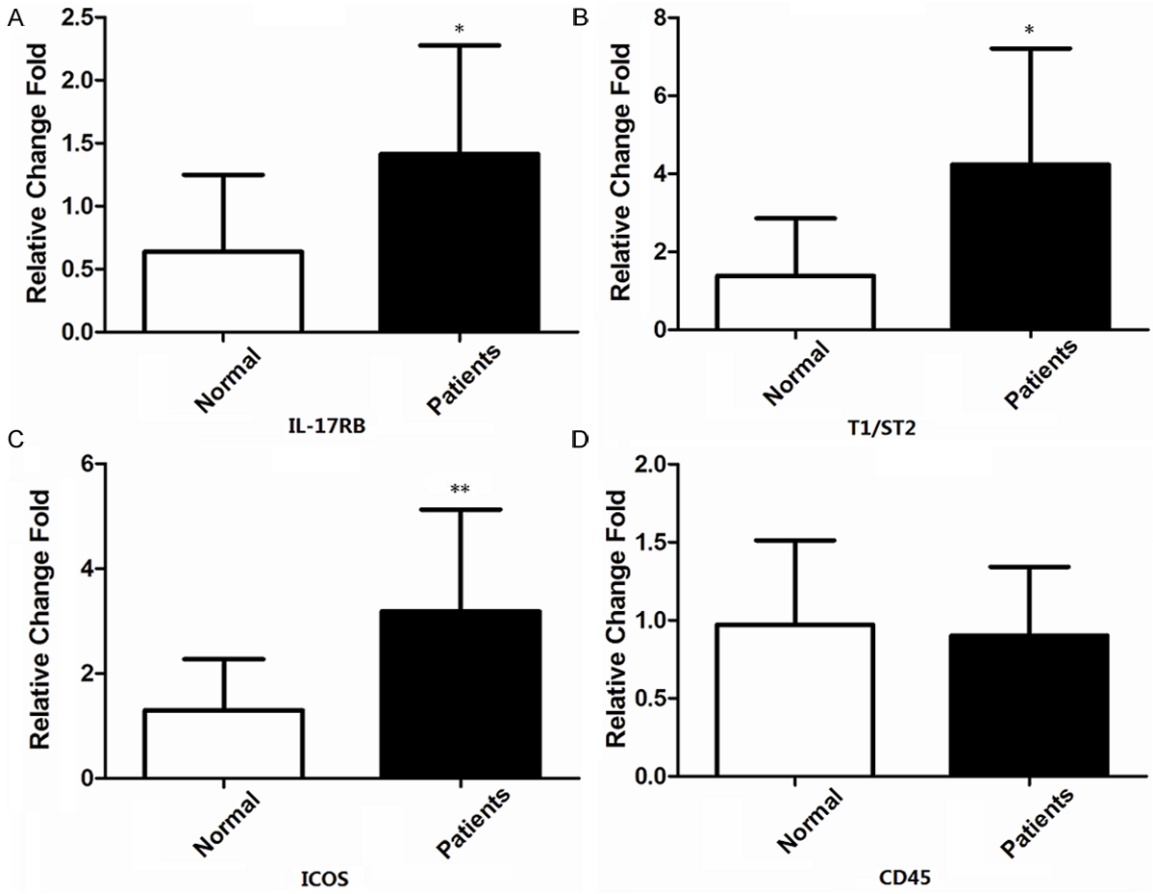

Figure 2. Levels of nuocytes associated receptors and surface markers. T1/ST2, IL-17RB, ICOS and CD45 mRNA in PBMC from GD patients were detected by qRT-PCR. The results of receptors analysis: IL-17RB (A) and T1/ST2 (B); the surface markers: ICOS (C) and CD45 (D). Data shown were represented as mean ± SD (All samples were measured in triplicate). $*P < 0.01$, $**P < 0.001$.

patients with Graves' hyperthyroidism, the transcription factors *RORα* mRNA in PBMC was detected. As shown in **Figure 1**, there was a trend of rising in the mRNA expression level of *RORα* in patients with graves' hyperthyroidism. At the same time, the transcription factors of *T-bet* and *GATA3* had thereby been changed correspondingly. Especially, the expression level of *T-bet* was obviously decreased (**Figure 1**).

Increased nuocytes relatively specific receptors and surface markers in PBMC from GD patients

Nuocytes express relatively specific receptors and surface markers including T1/ST2, IL-17RB, inducible T cell co-stimulator (ICOS) and CD45. We compared the mRNA levels of the relatively specific receptors and surface markers in PBMC from patients with those from healthy controls, and the results showed that the expression of T1/ST2, IL-17RB and ICOS were significantly increased in GD patients, while the level of CD45 mRNA was no significant change (**Figure 2**).

Different expression levels of nuocytes associated cytokines in PBMC or plasma from patients with Graves' hyperthyroidism

The qRT-PCR was used to analyze the levels of IL-13 and IL-5 mRNA in PBMC, and ELISA was performed to evaluate the levels of these signature cytokines in plasma. Our data indicated that the mRNA expression levels of IL-5 and IL-13 were significantly increased in PBMC from GD patients. And there was a trend of rising in these cytokines in plasma, but no statistical significance (**Figure 3**). In addition, nuocytes provide an innate source of Th2 cytokines, it may lead to Th1/Th2 dysequilibrium. In this

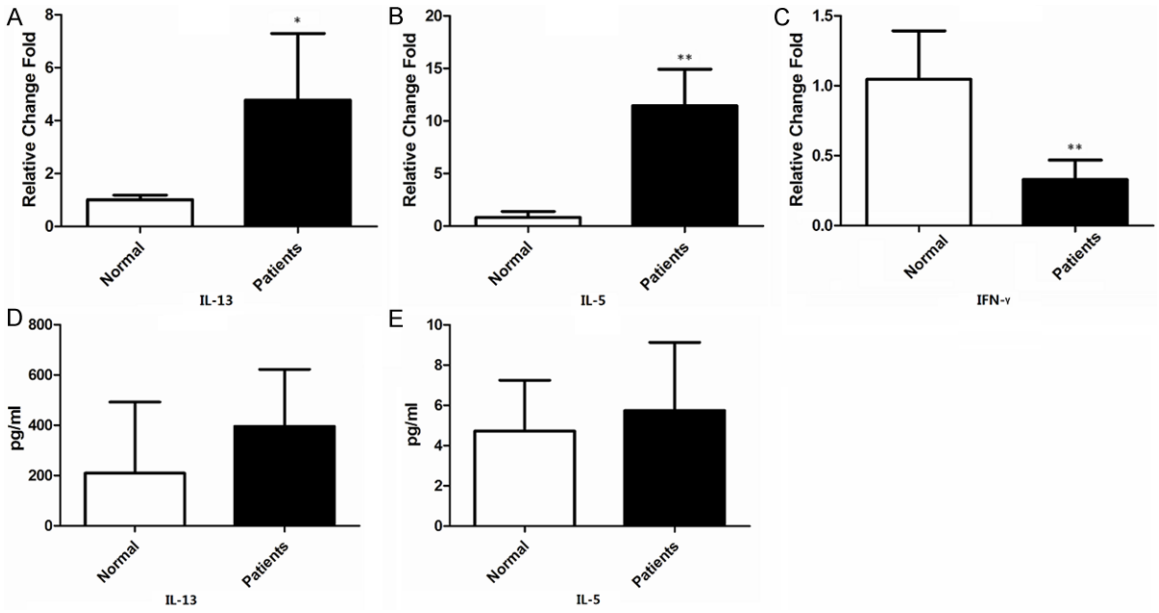

Figure 3. Enhanced levels of type 2 cytokines in Graves' hyperthyroidism. The qRT-PCR analysis of IL-13 (A), IL-5 (B) and IFN-γ (C) mRNA levels in PBMC from GD patients and healthy controls; ELISA analysis of IL-13 (D) and IL-5 (E) protein levels in plasma. Data shown are represented as mean ± SD (All samples were measured in triplicate). $*P < 0.05$, $**P < 0.01$.

experiment, we found that the mRNA expression level of IFN-γ was significantly decreased.

Increased frequency of nuocytes in the PBMC of patients with Graves' hyperthyroidism

The frequency of nuocytes in PBMC from Graves' hyperthyroidism patients or healthy volunteers was also determined by flow cytometry analysis. As shown in **Figure 4**, the frequency of nuocytes ($Lin^-ICOS^+IL\text{-}17RB^+$) was significantly elevated in Graves' hyperthyroidism patients compared with healthy volunteers ($P < 0.05$).

Correlation between the mRNA levels of IL-13 and IL-17RB or T1/ST2

IL-17RB and T1/ST2 are nuocytes relatively specific receptors or surface markers, and IL-13 is one of the nuocytes signature cytokines, they all involve in the differentiation and functional activity of nuocytes. In this study, we analyzed the correlation between IL-13 and T1/ST2 or IL-17RB in mRNA levels. The data indicated that there was a positive correlation between IL-13 and IL-17RB in Graves' hyperthyroidism patients, the positive correlation between IL-13 and T1/ST2 showed no significant differences (**Figure 5**).

Discussion

Autoimmune thyroid disease (AITD) is the most common organ-specific autoimmune disorder. AITD development occurs due to loss of immune tolerance and reactivity to thyroid autoantigens: thyroglobulin (TG) and thyroid stimulating hormone receptor (TSHR). This leads to infiltration of the gland by T cells and B cells that produce antibodies specific for clinical manifestations of hyperthyroidism in Graves' disease and chronic autoimmune thyroiditis (cAIT). In addition, T cells in Hashimoto's thyroiditis induce apoptosis in thyroid follicular cells, leading ultimately to the destruction of the gland. Cytokines are involved in the pathogenesis of thyroid diseases working in both the immune system and directly targeting the thyroid follicular cells. They are involved in the induction and effector phase of the immune response and inflammation, playing a key role in the pathogenesis of autoimmune thyroid disease. The presence of multiple cytokines has been demonstrated: IL-1α, IL-1b, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-14 and TNF-α within the inflammatory cells and thyroid follicular cells. Cytokines derived from T cells can directly damage thyroid cells, leading to functional disorders, thus increasing the inflammatory

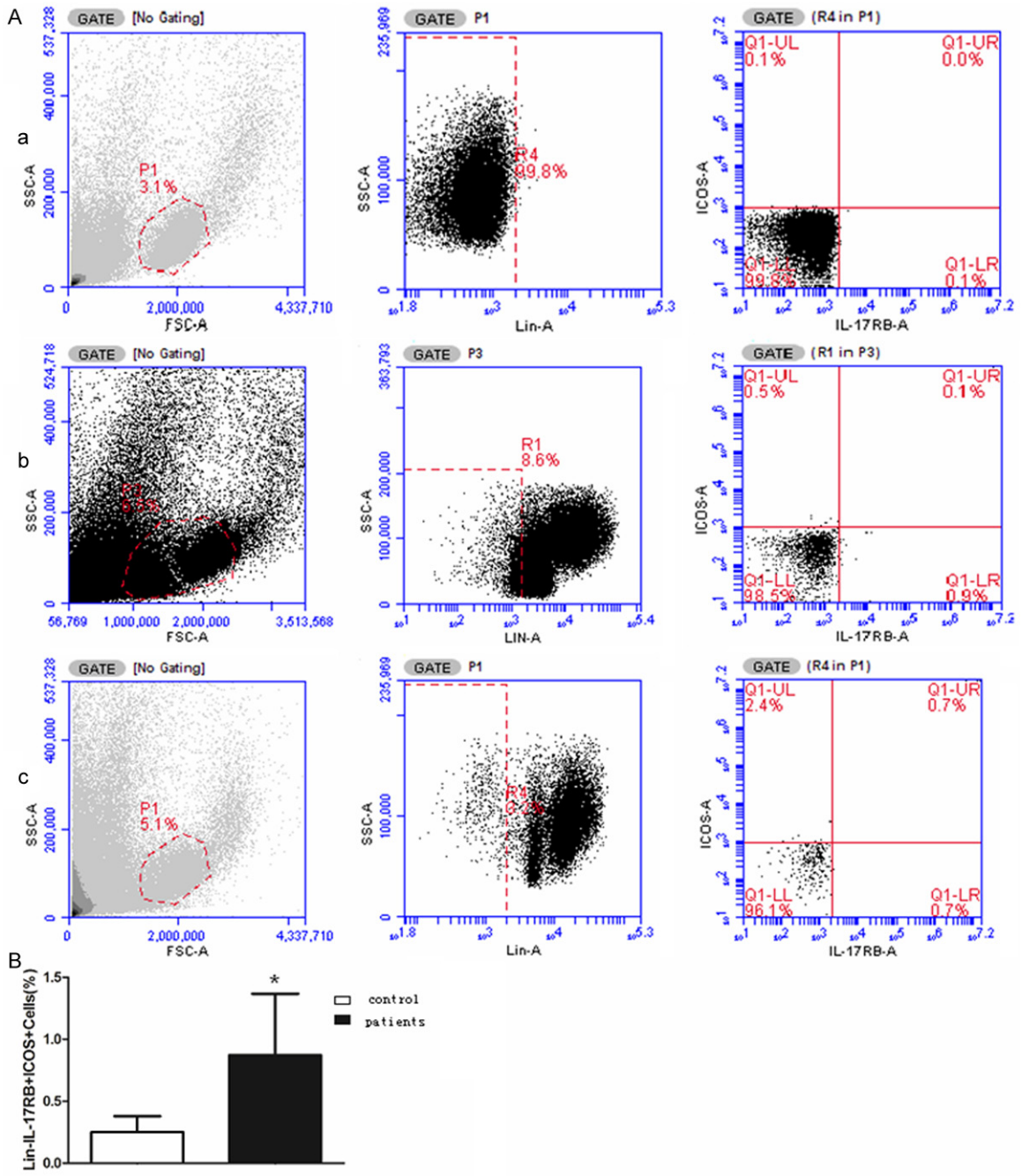

Figure 4. Enhanced nuocytes frequency in the PBMC from GD patients. The frequency of nuocytes in PBMC was analyzed by flow cytometry. (A) Representative diagrams of flow cytometry analysis for circulating nuocytes, (a) showed the result from healthy control specimens without specific antibody treatment; (b) indicated the result from healthy control specimens with antibody staining; (c) indicated the result from specimens of Graves' hyperthyroidism with antibody staining. (B) The frequency of nuocytes in PBMC from patients with Graves' hyperthyroidism was significantly increased compared with healthy controls ($P < 0.05$).

response in AITD. Immunological mechanisms involved in the pathogenesis of AITD are strongly related to each other, but differences in the image of chronic autoimmune thyroiditis cAIT and Graves' hyperthyroidism phenotype are possibly due to a different type of immune response observed in these two counteracting clinical thyroid diseases [14, 15]. This article described the potential role of nuocytes associated cytokines and surface markers or recep-

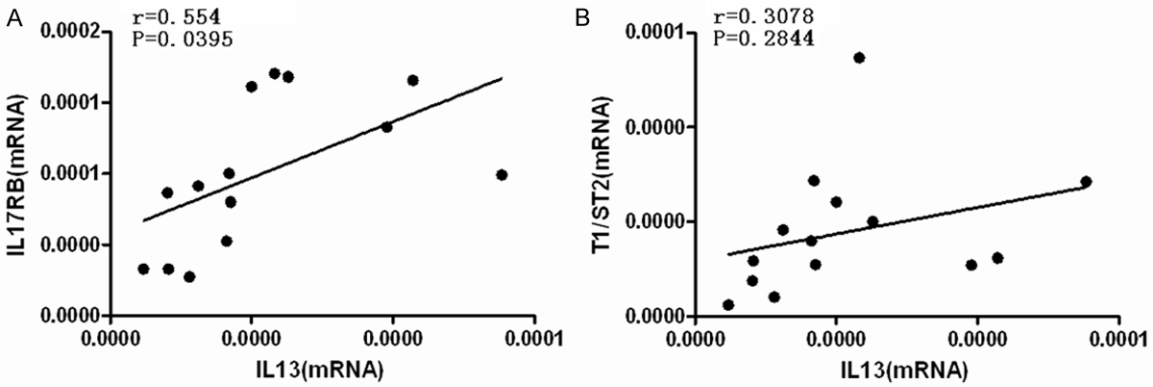

Figure 5. Correlation between IL-13 and IL-17RB or T1/ST2 mRNA levels. (A) The correlation of IL-13 and IL-17RB mRNA expression (r = 0.554, $P < 0.05$); (B) The correlation of IL-13 and T1/ST2 (r = 0.3078, $P > 0.05$). There was a positive correlation between IL-13 and IL-17RB in patients with Graves' hyperthyroidism.

tors in the pathogenesis of Graves' hyperthyroidism.

The recent identification of previously unrecognized nuocytes or natural helper cells has provided new insights into our understanding of the cellular mechanisms that lead to the development of $CD4^+$ Th2 cell-dependent immunity and/or inflammation at mucosal sites. This population is activated by IL-25 and/or IL-33 and expresses high levels of T1/ST2, IL-17BR, IL-7Rα, ICOS and CD45 [16, 17]. Nuocytes represent the predominant early source of IL-13 and IL-5 to promote and induce the development of Th2-type adaptive immune response including host resistance against parasitic helminth, airway inflammation and airways hyperreactivity in a murine model of allergic asthma, and are essential in the repair of damaged respiratory tissue following acute infection with influenza virus [18-23]. Recently, studies demonstrate that Th2 and/or Th17 responses have been associated with the pathogenesis of Graves' hyperthyroidism, while the role of nuocytes in hyperthyroidism has not been reported.

In present study, the nuocytes related genes or molecules in peripheral blood from patients with Graves' hyperthyroidism were measured, and the potential correlation between them was analyzed. The expression levels of T1/ST2, IL-17RB, ICOS, IL-5 and IL-13, which represented nuocytes associated molecules were significantly increased in patients, meanwhile, the *RORα* mRNA also had a tendency to increase. In addition, IFN-γ and *T-bet* (Th1 related cytokine and transcription factor) were obviously decreased. These results suggested that there were polarized nuocytes in Graves' hyperthyroidism patients, and which related to the down-regulation of Th1 cells or relatively advantage of Th2 differentiation. Th2 cells are characterized by the production of IL-4, IL-5, IL-9, and IL-13 and promote immunity to helminth infections and allergen-induced inflammation. Emerging studies indicate that the IL-25 (IL-17E) and IL-33 are critical in orchestrating distinct modules of the innate immune response that promote Th2 cells dependent immunity, inflammation and tissue repair. Recent studies have reported differential induction of nuocytes responses by IL-33 and IL-25, suggesting that these cytokines promote type 2 cytokine-dependent inflammation through distinct innate immune mechanisms [24-26]. Nuocytes can accept differential sources of stimulation through the distinct surface membrane molecule. The IL-1 family member, IL-33 promotes nuocytes by binding to T1/ST2 expressed on nuocytes, and the IL-17 family member, IL-25 is capable of promoting nuocytes through IL-17RB pathway. Our data showed that there was a positive correlation between IL-17RB and IL-13, which suggested that IL-17 may be the main way to promote nuocytes polarization in graves' hyperthyroidism. This was consistent with up-regulated Th17 found in the patients with Graves' hyperthyroidism.

In conclusion, the enhanced nuocytes was found in patients with Graves' hyperthyroidism through the detection of specific transcription factors, receptors and associated cytokines, which might participate in Th1 cells down-regulation or involve Th1/Th2 imbalance. In addi-

tion, the polarization of nuocytes was related to IL-25/IL-17RB pathway.

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Disclosure of conflict of interest

None.

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