ORIGINAL

The role of the IL-23/IL-17 axis in the pathogenesis of Graves' disease

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Abstract. This study is to explore the role of IL-23/IL-17 axis in subjects with Graves' disease, while IL-23/IL-17 axis plays an important role in a number of autoimmune diseases, but it's not clear in Graves' disease. Thirty-three patients with Graves' disease as a GD group, 15 patients with euthyroid GD as eGD group and 22 healthy volunteers as a control group whose age- and sex-matched. Peripheral blood was collected and peripheral blood mononuclear cells (PBMCs) were isolated in the both groups, then PBMCs were cultured in the presence or absence of IL-23 *in vitro*. The expression of retinoid-related orphan receptor gamma t (ROR γ t) and IL-17 mRNA were examined by Semi-quantitative RT-PCR, and the levels of IL-17 protein were measured by enzyme-linked immunosorbent assay. The expression of ROR γ t, IL-17 mRNA and IL-17 protein levels were markedly higher in GD and euthyroid GD group as compared with the control group. IL-17 levels were still higher in euthyroid GD patients. When PBMCs derived from the three groups were cultured *in vitro* with or without IL-23, the expression of ROR γ t in GD group with IL-23 dramatically increased as compared with that in GD group without IL-23 and in control group with IL-23. ROR γ t expression of PBMCs from eGD group cultured with IL-23 was increased compared with that cultured without IL-23. The levels of IL-17 mRNA and the protein were also significantly higher than that of GD and eGD cultured without IL-23 and control group. There was no difference of the expression of ROR γ t mRNA and IL-17 protein levels between GD and eGD group cultured with or without IL-23. Our studies demonstrated that IL-23/IL-17 axis is associated with the pathogenesis of Graves' disease in it activated term. This effect is not dependent on thyroid function, but may be associated to the immunity.

Key words: IL-23/IL-17 axis, Th17, IL-17, Graves' disease

GRAVES' DISEASE (GD) is one of the autoimmune diseases. It is generally believed that immune mechanism plays an important role in the pathogenesis of GD. The imbalance between Th1 and Th2 cells is implicated in the development of GD. Th17 cells, a newly discovered CD4⁺ T cell subset, and distinguished from the Th1 and Th2 cells, mainly produce IL-17 which acts *in vitro* and *in vivo* as a potent inflammatory cytokine [1]. It's functions reflected in the ability of the collective mobilization, recruitment and activation of neutrophils by the effectors they secreted [5, 6]. Th17 cells and IL-17 play an important role in various autoimmune diseases. Our previous findings [2, 3] and the latest study from Nanba [4] have shown that

Th17 cells and IL-17 were related to the pathogenesis of Graves' Disease, Hashimoto's thyroiditis (HT), and Graves' ophthalmopathy (GO). The retinoid-related orphan receptor gamma t (ROR γ t) is an important transcription factor in controlling the Th17 differentiation. ROR γ t can induce the expression of IL-17. When ROR γ t was knocked out, the incidence of autoimmune diseases was declined, and the number of Th17 cells was also corresponding reduced. [5-6].

IL-23, secreted by dendritic cells and macrophages, can activate and regulate autoimmune reactions and chronic inflammation which are induced by T cells. Recent studies have shown that IL-23 is required for full acquisition of the pathogenic function and maintenance of effector Th17 cells [7-9]. IL-23 receptors are widely presented on the surface of the memory T cells, NK cells, DCs cells, macrophages and microglia cells. IL-23 promotes the secretion of inflammatory factors, cytokines and chemokines *via* binding to IL-23

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receptor [10, 11]. The combination of IL-23 and IL-23 receptor may activate STAT3 signal, induced memory T cells to differentiate into Th17 cells and affected the expression of IL-17 by increasing the expression of ROR γ t, and, ultimately, promoted inflammation and autoimmune diseases [12].

IL-23/IL-17 axis mostly is composed of IL-23, IL-23 receptor, Th17 cells and IL-17. It's a critical pathway in activation and maintenance of Th17 cells. Studies found that the IL-23/IL-17 pathway is involved in the pathogenesis of rheumatoid arthritis, systemic lupus erythematosus, psoriasis, and other autoimmune diseases [13, 14]. However, it is still extremely unknown whether the IL-23/IL-17 axis is involved in the regulation of Graves' disease. In this study, we first explore the role of IL-23/IL-17 axis in Graves' disease. And this will help to elucidate the pathogenesis of Graves' disease.

Subjects and Methods

Material

Ficoll-Plaque (TBDsciences, China), IL-23, anti-CD3, anti-CD28 (Miltenyi Biotec, Germany), Total RNA Miniprep system, RT reagent Kit, Premix Taq Version 2.0 (Takara Biotechnology, China), Human IL-17 ELISA Kit (R&D, USA, the lower detection limit was 10 pg/mL).

Subjects

33 newly diagnosed patients with Graves' disease and 15 patients with euthyroid Graves' disease, who admitted in the First Affiliated Hospital of Chongqing Medical University from December 2011 to May 2012, were clinically and biochemically hyperthyroid respectively without other autoimmune and inflammation diseases. Informed consent was obtained from all participants, and the study was approved by the First

Affiliated Hospital of Chongqing Medical University Ethical Committee.

Graves' disease was diagnosed based upon the presence of diffuse goiter, the elevated serum thyroid hormones (FT4 and FT3) and suppressed TSH.

Patients with euthyroid Graves' disease was therapied by methimazol (MMI) for more than one year. Their thyroid functions are normal and now using MMI in a dose of 5-10mg qd.

Thyroid hormone and thyroid antibodies concentrations were determined by chemiluminescent assay using commercially available kits (normal ranges of free-triiodothyronine (FT3): 2.5-3.9 pg/mL, free-thyroxine (FT4): 0.61-1.12 ng/dL, thyrotropin (TSH): 0.35-3.5 uIU/L, TPOAb: 0-9 IU/mL, TGAb: 0-4 IU/mL). The clinical characteristics of the subjects were summarized in Table 1.

22 similar-aged healthy subjects were selected as the controls group. All of them, without any history of thyroid disease or autoimmune disease, were negative for thyroid-specific autoantibodies: anti-thyroperoxidase antibodies, TPOAb and anti-thyroglobulin antibodies, TgAb.

Blood samples

Plasma was separated immediately from fresh blood samples and stored at -80°C until analysis. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation over Ficoll-Hypaque cushions. Cell viability was always greater than 95%.

PBMC cultures and IL-23 stimulation in vitro

2×10^6 cells/mL PBMCs were planted in a 24-well flat-bottom tissue culture plate and maintained in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum at 37°C in a humidified atmosphere with 5% CO₂, then stimulated with 1 μ g/mL anti-CD3

Table 1 Thyroid hormones and auto-antibodies of different groups

	GD group	euthyroid GD group	control group
n (M/F)	33 (15/18)	15 (4/11)	22 (9/13)
Age (yr)	34.55 $\pm$ 10.60	34.87 $\pm$ 13.09	33.83 $\pm$ 10.04
FT3 (pg/mL)	12.67 $\pm$ 7.53*	2.93 $\pm$ 0.19	3.12 $\pm$ 0.31
FT4 (ng/dL)	6.93 $\pm$ 12.45*	0.81 $\pm$ 0.12	0.94 $\pm$ 0.34
uTSH (mIU/L)	0.06 $\pm$ 0.04*	2.15 $\pm$ 0.79	2.01 $\pm$ 0.91
TGAb (IU/mL)	69.16 $\pm$ 100.26*	22.85 $\pm$ 29.32*	<0.1
TPOAb (IU/mL)	146.08 $\pm$ 146.70*	57.88 $\pm$ 35.08*	0.93 $\pm$ 1.18

M: male; F: female; FT3: Free T3; FT4: Free T4; TGAb: thyroglobulin antibody; TPOAb: thyroperoxidase antibody;

*, $P < 0.05$ vs. control group.

and 1 µg/mL anti-CD28 antibodies in the presence or absence of IL-23 (20 ng/mL) respectively for 72 hours *in vitro*.

RNA isolation and Semi-quantitative RT-PCR detection of ROR γ t and IL-17

Total RNA was isolated with Trizol reagent, then used to synthesize cDNA with an RT Reagent Kit. Reverse-transcription PCR (RT-PCR) was performed with first-strand cDNA synthesized with 1 µg of total RNA and RT reagent Kit according to the manufacturer's instructions. The primers in the RT-PCR assays for Th17-specific transcription factor ROR γ t and IL-17 were designed by Primer Premier 5.0, as the following: ROR γ t, sense, 5'-CATTCAGTACGTGGTGGAGTTC-3'; antisense, 5'-GAGAACAAGGGCTGTGTAGAGG-3'; IL-17, sense, 5'-TGGTGTCACTGCTACTGCTGC-3'; antisense, 5'-TTGCTGGATGGGGACAGAGTT-3'. Reference primer of β -actin was the following, sense, 5'-GACCCAGATCATGTTTGAGACC-3'; antisense, 5'-ATCTCCTTCTGCATCCTGTCG-3'. Human β -actin, a reference gene, was used to normalize each sample and each gene. Prepared cDNA was used for PCR amplification with the above primers under the following conditions: pre-heating at 94°C for 5 min, denaturing at 94°C for 30 sec, annealing at 55°C (ROR γ t), 60°C (IL-17) or 55°C (β -actin) for 30 sec, and extension at 72°C for 30 sec. The reaction repeated for 35 cycles followed by incubation at 72°C for 5 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing 1 µl Goldview. The resulting bands were observed and photographed under ultraviolet light and measured using the Digital Gel Imaging Analyst (BIO-RAD CheniDOC XRS, USA). Density was determined for each sample PCR product, including the positive control. Background density was subtracted from each band, and the relative values of ROR γ t and IL-17 mRNA were calculated using β -actin mRNA as a standard. PCR products were sequenced by Takara Biotechnology (Dalian) Co., Ltd (China), and blasted in the NCBI Blast bank.

Enzymeimmunoassay (ELISA)

IL-17 in culture supernatants were detected respectively using the Human IL-17 ELISA Kit.

Statistical analysis

The statistical significance was estimated by SPSS13.0. All data were expressed as mean $\pm$ SD.

Differences within two groups were compared by paired *t*-test (*t*²-test). Differences among between examined groups were performed using one-way ANOVA analysis of variance followed by S-N-K (equal variances assumed) or Games-Howell (equal variances not assumed) *post-hoc* test. A value of *P* < 0.05 was considered to be statistically significant.

Results

ROR γ t and IL-17 mRNA expression and IL-17 protein levels were significantly elevated in GD and euthyroid GD patients

The expression of the Th17-specific transcription factor ROR γ t and IL-17 mRNA were dramatically elevated in PBMCs of GD and euthyroid GD group cultured without IL-23 for 72 hours as compared with control group (Fig. 1 and Fig. 2). IL-17 protein level was significantly increased in GD and euthyroid GD group cultured without IL-23 for 72 hours compared to the control group. And there was no difference of ROR γ t mRNA and IL-17 protein level between GD and euthyroid GD group. As demonstrated in Table 2.

ROR γ t and IL-17 mRNA expression and IL-17 protein levels were increased by IL-23 stimulation

In GD group, the PBMCs stimulated by IL-23 (20 ng/mL) for 72 hours, ROR γ t and IL-17 mRNA expression were significantly increased; IL-17 protein levels in the supernatants of the cultured cells was also significantly increased. The increased differences for ROR γ t and IL-17 mRNA expression and IL-17 protein levels between the groups with and without IL-23 are statistically noticeably significant (*P* < 0.01), and even more significant between the GD group and control group (*P* < 0.05). In euthyroid GD group, the PBMCs stimulated by IL-23 (20 ng/mL) for 72 hours, ROR γ t mRNA expression were significantly increased; IL-17 protein levels in the supernatants of the cultured cells was also significantly increased, and ROR γ t, IL-17 mRNA expression and IL-17 protein levels were significantly increased compared with the control group. No difference of ROR γ t, IL-17 mRNA expression and IL-17 protein levels was found between GD and euthyroid GD group. [Figs. 1, 2 and Table 2]. Those results supported that IL-23/IL-17 plays an important role in human Graves' disease, and this effect is not dependent on thyroid function, which may be associated to the immunity.

Fig. 1 RORγt mRNA expression in PBMCs of the two groups cultured in the presence or absence of IL-23. Increased induction of RORγt gene in PBMCs from GD and eGD patients. A, PBMCs from 33 patients with GD, 15 patients with eGD and 22 people of control group were cultured in the presence or absence of IL-23, and then the levels of RORC2 mRNA were determined by Semi-quantitative RT-PCR. Data from five representative patients, two with GD, two with eGD and one from control group, are shown. B, Detection of RORγt mRNA in PBMCs from GD, eGD patients and control group after cell cultured in the presence or absence of IL-23. RORγt mRNA was estimated by Semi-quantitative RT-PCR. Data correspond to the arithmetic mean±SEM of 33 patients with GD, 15 patients with eGD and 22 people of control group. A: 1, 3: GD+IL-23; 2, 4: GD; 5, 7: eGD+IL-23; 6, 8: eGD; 9: NC+IL-23; 10: NC. RORγt: 295bp; β-actin: 594bp. *, $P < 0.05$ vs. control group; #, $P < 0.05$ vs. GD group; †, $P < 0.05$ vs. eGD group.

Fig. 2 IL-17 mRNA expression in PBMCs of the two groups cultured in the presence or absence of IL-23. Increased induction of IL-17 gene in PBMCs from GD and eGD patients. A, PBMCs from 33 patients with GD, 15 patients with eGD and 22 people of control group were cultured in the presence or absence of IL-23, and then the levels of IL-17 mRNA were determined by RT-PCR. Data from five representative patients, two with GD, two with eGD and one from control group, are shown. B, Detection of IL-17 mRNA in PBMCs from GD, eGD patients and control group after cell cultured in the presence or absence of IL-23. IL-17 mRNA was estimated by Semi-quantitative RT-PCR. Data correspond to the arithmetic mean±SEM of 33 patients with GD, 15 patients with eGD and 22 people of control group. A: 1, 3: GD+IL-23; 2, 4: GD; 5, 7: eGD+IL-23; 6, 8: eGD; 9: NC+IL-23; 10: NC. IL-17: 329bp; β-actin: 594bp. *, $P < 0.05$ vs. control group; #, $P < 0.05$ vs. GD group.

Table 2 IL-17 levels of different groups (pg/mL)

	GD group	euthyroid GD group	control group
present of IL-23	376.91±67.93*#	371.73±31.73*#	317.59±57.50#
absent of IL-23	319.82±76.41*	299.66±35.49*	238.80±57.05

Increased IL-17 levels of GD group. PBMCs from 33 patients with GD, 15 patients with euthyroid GD and 22 people of control group were cultured in the presence or absence of IL-23, and then the levels of IL-17 in culture supernatants were determined by ELISA. Data correspond to the arithmetic mean±SEM of 33 patients with GD, 15 patients with eGD and 22 people of control group. Differences within two groups were compared by paired t-test (*t'*-test). Differences between examined groups were performed using one-way ANOVA analysis of variance followed by S-N-K (equal variances assumed) or Games-Howell (equal variances not assumed) *post-hoc* test. A value of $P < 0.05$ was considered to be statistically significant. *, $P < 0.05$ vs. control group; #, $P < 0.05$ vs. absent of IL-23 group.

Discussion

IL-23/IL-17 axis plays an important role in inflammation and various autoimmune diseases, but its role in the pathogenesis of GD is not clear. In this study, we found that ROR γ t mRNA, IL-17 mRNA and the protein expression were significantly higher in GD patients. It shows that an imbalance of Th17 cells and IL-17 levels may be involved in GD patients, and associated with the pathogenesis of GD. Th17 cells can induce epithelial cells and antigen presenting cells to produce IL-1, IL-6, IL-8, TNF and other cytokines by secreting IL-17, ultimately lead to the development of GD. It can be seen that Th17 cells and IL-17 may play an important role in the pathogenesis of autoimmune diseases, including Graves' disease.

The newly discovered Th17 cells, a new class of CD4⁺ T cell subsets, also have an important regulatory role in autoimmune diseases and infectious diseases [15]. Such cells can compensate the inadequacies of Th1/Th2 mediated effect mechanisms. Although Th17 cells secrete IL-17, IL-17F, IL-22 and other cytokines, its main function is the secretion of IL-17. Several cytokines are involved in the differentiation of human Th17 cells [16-19]. TGF- β and IL-6 play a key role in the Th17 differentiation, they induce CD4⁺ T cells differentiate to the Th17 cells by the activation of ROR γ t [20]. IL-23 plays a crucial role in maintaining the Th17 differentiation and promoting IL-17 secretion [21]. IL-23 plays a key role in the pathogenesis of autoimmune diseases. IL-23 can lead to accumulate of Th17 cells in inflamed tissues. When lacked of IL-23, the generation of Th17 cells and their cytokines will be reduced [22]. Obviously, IL-23 is important for Th17 cells to adapt internal environment and secrete cytokines. In this study, we cultured PBMCs of GD

patients and normal controls with IL-23 *in vitro*. We found that ROR γ t and IL-17 mRNA expression in the cultured cells and IL-17 protein levels in the culture supernatants of GD group increased after IL-23 stimulation compared with PBMCs of GD group cultured without IL-23. At the same time, the same increases in GD group were also significant as compared with control group. This is because IL-23 may combine with IL-23 receptor on the cell surface, which enhances IL-6, IL-1 β and TNF- α expression through the positive feedback loop, promotes Th17 cell differentiation and IL-17 secretion, and leads to the pathogenesis of GD [21]. In addition, IL-17 from the innate immune can combine with IL-17 receptor which is on the surface of dendritic cell (DC), then the combination promotes DCs secrete large amounts of IL-23, and further promotes the differentiation of Th17 cells and IL-17 secretion [23]. This shows that the IL-23/IL-17 axis may be associated with the pathogenesis of GD, and play an important role in the pathogenesis of GD.

In the euthyroid GD group, the level of FT3, FT4 and uTSH were normal, but the level of TPOAb and TgAb which were related to the immunity were still higher than the control group. In this study, we found that the ROR γ t mRNA expression and IL-17 protein levels were significantly increased in the euthyroid GD group compared with the control group when cultured without IL-23. But there was no difference between GD and euthyroid GD group. Figueroa-Vega *et al.* [24] found that although the thyroid function were lower in HT patients and higher in GD patients, a increased proportion of Th17 cells and enhanced levels of IL-17 mRNA in the peripheral blood in patients with HT and GD compared with health control were detected, and a greater Th17 differentiation effect was observed in cells stimulated with IL-23, which was higher in HT and GD

patients compared with healthy controls. Nanba *et al.* [4] found the proportion of Th17 cells were significantly higher in intractable GD patients than those in patients with GD in remission, although the thyroid function of intractable GD and GD in remission were both in the normal range. This indicates that the elevation of ROR γ t and IL-17 mRNA expression and IL-17 protein levels is not dependent on thyroid function, and may be more associated to the immunity. When cultured with IL-23 for 72 hours, ROR γ t and IL-17 mRNA expression and IL-17 protein levels were significantly increased. It shows that IL-23/IL-17 axis may be involved in the pathogenesis of GD whether thyrotoxicosis or euthyroid, and may be more associated with immunity. Interestingly, we found significant increase of IL-17 mRNA in GD+IL-23 group compared with GD group, and we also found that IL-17 mRNA expression was not different between eGD and eGD+IL-23 groups. There only a upward trend between eGD and eGD+IL-23 groups. This may because of we have studied only a small number of these patients.

The role of IL-23/IL-17 axis in Graves' disease has not been reported so far, but studies showed that IL-23 and IL-17 levels elevated in the peripheral blood of patients with GD. Nanba *et al.* [4] also found that Th17 lymphocyte levels were higher in GD patients as compared with healthy controls, and those patients with intractable GD showed a higher proportion of Th17 cells than patients with GD in remission. In addition, studies have found that IL-23 receptor gene polymorphisms were closely related to Graves' ophthalmopathy [25]. These data support that the IL-23/IL-17 axis may be involved in the pathogenesis of GD. However, these studies were only simple observation about the phenomenon and without IL-23 stimulation. When Figueroa-Vega *et al.* [24] explored the role of Th17

cells in AITD, they found that after the IL-23 stimulation, the proportion of Th17 cells and IL-17 levels were elevated in GD patients who had relapsed after antithyroid drug treatment and were euthyroid under carbimazole therapy at the time of surgery (two of these patients had ophthalmopathy) compared with health controls, but no difference on RORC₂ expression between the two groups. It is feasible that the discrepancies between this study and ours result from, in part, the different genetic background of the individuals studied, and the stage of the disease of the patients.

In conclusion, we found that the IL-23/IL-17 axis may participate in the pathogenesis of GD, and play an important role in GD. Further studies are needed to clarify the specific mechanism of IL-23/IL-17 axis in the pathogenesis of GD. Our study provides a new direction and research basis for the further studies of the pathogenesis of GD, and also supplies a new potential target for the treatment of GD. And this effect is not dependent on thyroid function, but may be associated to the immunity.

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Disclosure Summary

The authors has no a financial relationship with a commercial entitle that has an interest in the subject of the manuscript.

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