RESEARCH

Serum concentrations of TNF-α and its soluble receptors in Graves' disease

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Abstract

Graves' disease (GD), an organ-specific autoimmune disease, is the most common cause of hyperthyroidism. Tumour necrosis factor-alpha (TNF-α) exhibits immunological and metabolic activities involved in the induction and maintenance of immune responses. We attempted to evaluate the relationship between GD and serum TNF-α and its soluble receptors (sTNFRs), soluble TNF receptor 1 and 2 (sTNF-R1 and sTNF-R2). A total of 72 GD patients and 72 matched healthy individuals were recruited for this study. Serum TNF-α and sTNFRs were measured by sandwich ELISA. In our study, no significant difference was observed in TNF-α, but sTNFRs were found to be significantly elevated in GD patients compared to healthy individuals. Serum sTNFR levels were positively correlated with free triiodothyronine (FT3) and free thyroxine (FT4), and TNF-α was negatively correlated with thyroid-stimulating hormone (TSH) in the GD group. It was also shown that thyrotropin receptor antibody (TRAb) was positively correlated with TNF-α and sTNFRs. Spearman's correlation analysis showed that only sTNF-R1 was positively correlated with complement C3. Multiple linear regression analysis suggests that serum levels of sTNF-R1 and FT4 may play an important role in the serum level of FT3. According to the median value of FT3 level, GD patients were further divided into a high FT3 group and a low FT3 group. The serum levels of sTNF-R1 in the high FT3 GD group were significantly higher than those in the low FT3 GD group. In conclusion, sTNFRs may play an important role in anti-inflammatory and immune response in GD.

Key Words
- TNF-α
- sTNF-R1
- sTNF-R2
- Graves' disease
- immune

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Introduction

Graves' disease (GD) is an organ-specific autoimmune disease that is characterised by the infiltration of thyroid antigen-specific T-cells into thyroid-stimulating hormone receptor-expressing tissues (1). Its pathogenesis is still unclear and may be related to genetics, environment, immunity and other factors, among which immune response is the core factor.

It is recognised that the autoimmune process may have a very important pathogenic role in Graves' disease (2). A proinflammatory cytokine, TNF-alpha (TNF-α), exhibits immunological and metabolic activities involved in the induction and maintenance of immune responses. In the thyroid gland, the proinflammatory cytokine may exert its functions in cooperation with infiltrating immunocompetent cells (3, 4). In recent years, scholars have devoted more attention to the abnormal expression of cytokines in Graves' disease. Cytokines can affect the growth and differentiation of thyroid follicular cells, leading to the abnormal synthesis and secretion of thyroid hormones and thyroid function disorder.

TNF-α is a pleiotropic inflammatory cytokine produced and secreted mainly by monocytic cells, lymphocytes and other cell types such as thyroid epithelial cells and fibroblasts within the thyroid gland (5, 6). TNF-α elicits

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its biological activities by binding to its two types of specific receptors, which have been identified in the thyroid follicular cells of humans and animals (7). Soluble receptors for TNF-α can be detected in the body fluids of patients. They are generally produced through the hydrolysis of membrane receptor protease and then shed from the surface of the cell membrane in the extracellular region. They are also secreted extracellularly by different splicing methods in the production of the receptor for mRNA (8). The soluble forms of TNF receptors (sTNFRs) represent the extracellular domains of these proteins with molecular weights of 55 kDa (soluble TNF receptor 1, sTNF-R1) and 75 kDa (soluble TNF receptor 2, sTNF-R2). The biological activity of TNF-α is mediated principally by TNF-R1, while TNF-R2 is used for signal transduction (9).

As TNF-α is usually short-lived in the peripheral blood, controversy has been generated by conflicting reports of serum levels of TNF-α being within the normal range (10, 11, 12) or elevated (13) or depressed (14) in patients with hyperthyroidism. The receptors for TNF (TNFRs) are shed from cells and transform into sTNFRs, which can be measured in the peripheral blood. Recently, it has been confirmed that TNFRs exhibit longer half-lives in the peripheral blood than that of TNF-α itself. The serum concentrations of sTNFRs can be sensitive indicators of the activation of the TNFR-TNF-α system (15, 16).

In this study, we investigated the levels of TNF-α, sTNF-R1 and sTNF-R2 in the serum of patients with GD and attempted to determine the risk factors that may affect the occurrence and development of Graves' disease.

Patients and methods

Patients

A total of 72 GD patients without Graves' ophthalmopathy (52 females and 20 males, mean age 41 ± 11 years) were recruited for this study at the Nantong City No 1 People's Hospital and Second Affiliated Hospital of Nantong University between June 2018 and January 2019. Sixty of them were newly diagnosed GD patients. Twelve of them presented a recurrence of a previously diagnosed Graves' disease.

Inclusion criteria

The diagnosis of Graves' disease was based on the WHO criteria (17). Graves' disease patients often have one or more of the following characteristics:

a. Thyrotoxicosis (confirmed by the determination of thyroid hormone and signs of hyperthyroidism);
b. Goitre (confirmed by palpation and B-ultrasound);
c. Ophthalmopathy (manifested as exophthalmos or other invasive ophthalmic signs);
d. Dermopathy (pretibial myxoedema).

Diagnosis

a. Symptoms and signs: goitre, exophthalmos or disorders of eye movement, menstrual disorders;
b. Laboratory examination: increases in serum total thyroxine (T4), triiodothyronine (T3), free thyroxine (FT4), free triiodothyronine (FT3), reverse T3 (rT3); declines in thyroid-stimulating hormone (TSH); and thyroid-stimulating antibody (TSAb) and other related antibodies can be detected.

Among these, signs of hyperthyroidism, diffuse goitre of thyroid, decrease in serum TSH and increase of thyroid hormone are essential diagnostic conditions.

Exclusion criteria

a. With Graves' ophthalmopathy;
b. Drug administration history in the past 3 months, especially antithyroid drugs, antibiotics and immunomodulators;
c. Pregnancy, tumour or infection (criteria of no infection: (a) no obvious infected focus; (b) leukocyte count: 4–10 × 10^9/L, lymphocyte percentage: 20–40%, and neutrophil percentage: 50–70%; and (c) axillary temperature: 36–37°C);
d. History of other autoimmune diseases and renal insufficiency or family history of other autoimmune diseases.

Another 72 euthyroid healthy controls (HC) (47 females and 25 males, mean age 39 ± 9 years) without clinical evidence or a family history of any autoimmune diseases were selected to participate in this study after recording histories, physical examination and confirmation of the normal FT3, FT4, TSH and negative thyroid autoantibodies.

All the participants were Chinese. Informed consent was obtained from all participants. The study was approved by the institution review board of Nantong City No 1 People's Hospital and Second Affiliated Hospital of Nantong University and complied with the Declaration of Helsinki.

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Methods

Blood samples were drawn after an overnight fast and clotted in serum separator tubes (SSTs) for 30 min. After sufficient clotting, samples were centrifuged at 1000 ***g*** for 15 min, and serum was removed from SSTs and then stored at −80°C in preparation for the assays. Serum TNF-α and its soluble receptors were measured by the quantitative technique of a 'sandwich' ELISA. Circulating TNF-α levels were detected by sandwich ELISA (Multi Sciences (Lianke) Biotech, Co., Ltd, Hangzhou, Zhejiang Province, China). Meanwhile, the human sTNF-R1 and sTNF-R2 concentrations were measured by 'sandwich' ELISA (ImmunoWay Biotechnology Company, Plano, TX, USA). This immunoassay method utilises the quantitative technique of a 'sandwich' ELISA, where the target protein (antigen) is bound in a 'sandwich' formation by the primary capture antibodies coated to each well-bottom and the secondary detection antibodies subsequently added by the researcher. The capture antibodies coated to the bottom of each well are specific for a particular epitope, while researcher-added detection antibodies bind to epitopes on the captured target proteins. The absorbance of each well can be detected with a spectrophotometer, allowing for the generation of a standard curve and subsequent determination of protein concentration. The range of the standard curves were 0.42–1000, 15.625–1000 and 8–500 pg/mL, respectively. BMI was calculated as the weight (kg)/the height (m) squared. Blood pressure (BP) was measured in triplicate after at least 30 min of rest, and the average of the three recordings was used for the analysis. According to the ultrasonographic investigation of the thyroid gland, the volume of each lobe for each patient was calculated by the formula: V (mL) = 0.479 × depth × width × length (cm). Thyroid VolT was calculated as the sum of the volume of the two lobes (18). Meanwhile, the ultrasonographic investigation of the thyroid glands in the healthy control group determined that the thyroids in these patients were not enlarged.

Statistical analysis

Skewness and kurtosis tests were used to assess the normality of the distribution. Normally distributed data were reported as the mean±S.D. Data that did not show a Gaussian distribution were expressed as median (interquartile range). Differences between two groups were evaluated using the Mann–Whitney *U*-test when the data did not show normal distribution. The correlations of serum TNF-α and its soluble receptor levels with other parameters were analysed using Spearman's Rho test. Multiple linear regression was used to study the effects of clinical parameters and the level of soluble TNF receptor on FT3. Analyses were performed using SPSS version 25.0 and GraphPad Prism version 8.0 for Windows. Differences were considered to be statistically significant if P values were <0.05.

Results

Characteristics of GD patients and healthy controls

The age (P=0.193) and sex (P=0.369) distributions were similar between GD patients and healthy controls. The BMI values of patients were significantly lower than for normal controls (P<0.001). Individuals with Graves' disease had significantly higher levels of FT3 (17.7 (9.64–30.7) pmol/L, normal values 3.28–6.47 pmol/L) and FT4 (46.7 (23.6–61.5) pmol/L, normal values 7.64–16.0 pmol/L) and lower TSH (0.01 (0.01–0.02) mIu/L, normal values 0.49–4.91 mIu/L) concentrations than did controls (P<0.001). Thyrotropin receptor antibody (TRAb) was found elevated to 7.22 (3.77–18.4) IU/L (normal values are below 1.58 IU/L) in patients compared to normal subjects (P<0.001). It was also found that levels of thyroid peroxidase antibody (TPOAb) (137 (9.40–976) IU/mL, normal values are below 9 IU/mL) and thyroglobulin antibody (TgAb) (2.25 (0.40–79.8) IU/mL, normal values are below 4.9 IU/mL) were significantly higher in GD patients than in normal controls (P<0.001). In comparison with healthy subjects, the total thyroid gland volume (Thyroid VolT) of GD patients was increased to 16.9 (12.5–22.5) mL as assessed by sonography. The detailed characteristics of the subjects are shown in Table 1.

Serum TNF-α, sTNF-R1 and sTNF-R2 levels in GD and HC groups

As shown in Table 2, serum TNF-α levels were 39.0 (6.18–121) pg/mL for GD patients and 23.6 (4.99–72.0) pg/mL for the controls, but there were no significant differences between these two groups (P=0.25). The GD group (33.6 (19.8–52.1) pg/mL) exhibited a significantly higher serum concentration of sTNF-R1 than that of the healthy group (25.8 (17.3–39.0) pg/mL) (P=0.037). Similarly, the concentration of sTNF-R2 was greater in GD patients (24.6 (13.8–38.7) pg/mL) than in control subjects (14.0 (8.17–29.9) pg/mL) (P =0.001). The detailed distributions are shown in Fig. 1.

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Table 1 Characteristics of GD patients and healthy controls.

Variables	GD	HC	*P*
n	72	72	–
Age (year)	41 ± 11	39 ± 9	0.193
Sex (F/M)	52/20	47/25	0.369
Smoking (Y/N)	16/56	19/53	0.596
BMI (kg/m^2)	21.5 ± 3.52	23.7 ± 3.30	**<0.001**
SBP (mmHg)	131 ± 16	115 ± 14	**<0.001**
DBP (mmHg)	77 ± 8	77 ± 11	0.716
Duration (month)	2(0.5–12)	–	–
FT3 (pmol/L)	17.7(9.64–30.7)	5.17(4.75–5.77)	**<0.001**
FT4 (pmol/L)	46.7(23.6–61.5)	10.2(9.11–11.4)	**<0.001**
TSH (mIu/L)	0.01(0.01–0.02)	1.91(1.29–2.71)	**<0.001**
TRAb (IU/L)	7.22(3.77–18.4)	0.30(0.30–0.40)	**<0.001**
TPOAb (IU/mL)	137(9.40–976)	0.50(0.30–1.25)	**<0.001**
TgAb (IU/mL)	2.25(0.40–79.8)	0.10(0.10–0.25)	**<0.001**
Thyroid VolT (mL)	16.9(12.5–22.5)	*n*	–
IgG (g/L)	14.1 ± 3.88	13.3 ± 2.19	0.562
IgM (g/L)	1.19 ± 0.57	1.36 ± 0.58	0.171
IgA (g/L)	2.02(1.61–2.69)	2.16(1.64–2.95)	0.311
C3 (g/L)	1.30(1.03–1.47)	1.21(1.03–1.58)	0.582
C4 (g/L)	0.33(0.29–0.41)	0.29(0.23–0.33)	**0.004**
CD3 (/μL)	1182 ± 406	1428 ± 403	**0.026**
CD4 (/μL)	666 ± 229	708 ± 226	0.660
CD8 (/μL)	484 ± 202	714 ± 240	**<0.001**
CD19 (/μL)	242(187–320)	240(189–324)	0.900
CD16 + 56 (/μL)	255(168–330)	352(268–561)	**0.001**
CD3 (%)	67.1 ± 7.88	66.7 ± 9.17	0.916
CD4 (%)	38.0 ± 6.01	33.0 ± 5.57	**0.001**
CD8 (%)	27.3 ± 6.55	33.3 ± 7.97	**0.002**
CD19 (%)	16.6 ± 6.33	12.5 ± 4.29	**0.004**
CD16 + 56 (%)	15.0(11.0–20.0)	16.0(13.0–29.5)	0.081

Normally distributed values in the table are mean ± S.D. and non-normally distributed values are median (25 and 75% interquartiles).
C3/4, complement C3/4; F/M, female/male; FT3, free triiodothyronine; FT4, free thyroxine; GD, Graves' disease; HC, healthy control; IgG/M/A, immunoglobulin G/M/A; *n*, number of patients; SBP/DBP, systolic/diastolic blood pressure; TgAb, thyroglobulin antibody; thyroid VolT, total thyroid gland volume; TPOAb, thyroid peroxidase antibody; TRAb, hyrotropin receptor antibody; TSH, thyroid-stimulating hormone; Y/N, yes/no. Bold indicates statistical significance, $P < 0.05$.

According to the median values for FT3, GD patients were further divided into a high FT3 GD group and a low FT3 GD group

Based on the median of values for FT3 (17.7 pmol/L) levels, GD patients were further divided into a high FT3 GD group (FT3 > 17.7 pmol/L) and a low FT3 GD group (FT3 < 17.7 pmol/L). The clinical parameters for each group are shown in Table 3. There were no significant differences in age (P=0.585), gender (P=0.293) distribution, BMI (P=0.274), SBP (P=0.674), DBP (P=0.706) or course of disease (P=0.793) between the high FT3 GD group and the low FT3 GD group. Compared with the low FT3 GD group, the thyroid volume (P=0.002), thyroid antibody TRAb (P=0.007) level, and immune index CD16+56 (P=0.048) increased significantly, while values of the immune index IgM (P=0.046) decreased significantly in the high FT3 GD group. The serum levels of sTNF-R1 (P=0.008) in patients in the high FT3 GD group were significantly higher than those in the low FT3 GD group, but there was no significant difference for levels of TNF-α (P=0.150) and sTNF-R2 (P=0.126).

Table 2 Serum TNF-α and sTNFRs levels in GD and HC groups.

Variables	GD	HC	*P*
TNF-α (pg/mL)	39.0 (6.18–121)	23.6 (4.99–72.0)	0.25
sTNF-R1 (pg/mL)	33.6 (19.8–52.1)	25.8 (17.3–39.0)	**0.037**
sTNF-R2 (pg/mL)	24.6 (13.8–38.7)	14.0 (8.17–29.9)	**0.001**

Correlations between serum TNF-α and its soluble receptor levels and related thyroid parameters in GD patients

There was strong correlation between the serum levels of sTNF-R1 and sTNF-R2 (Fig. 2). When the sTNF-R1 and

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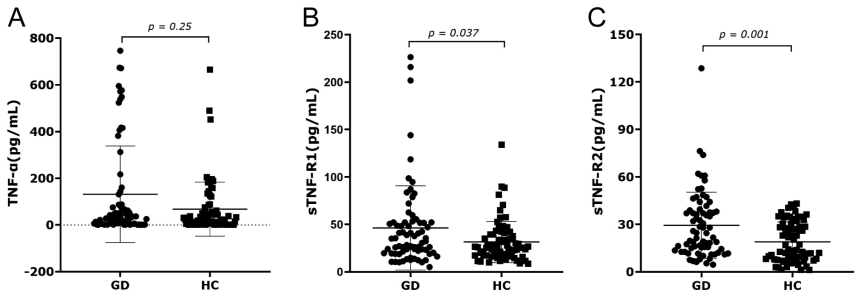

Figure 1
Serum TNF-α (A), sTNF-R1 (B) and sTNF-R2 (C) levels in GD patients and healthy controls.

sTNF-R2 levels were positively correlated with indices of thyroid function such as FT3 (P=0.001, P=0.012, respectively, Fig. 3B and C) and FT4 (P=0.024, P=0.028, respectively, Fig. 3E and F) within the patient group, TNF-α was negatively correlated with TSH (P=0.024, Fig. 3G). It was also found that TRAb level was positively correlated with levels of TNF-α, sTNF-R1 and sTNF-R2 (P=0.024, P=0.010, P=0.030, respectively, Fig. 4G, H, I and Table 4). In addition, we did not find any correlations among levels of TPOAb, TgAb and TNF-α and its soluble receptors (Fig. 4A, B, C, D, E, F and Table 4). Similarly, the inflammatory cytokines we measured were not correlated with either Thyroid VolT or duration.

Correlations between serum TNF-α and its soluble receptor levels and related immune parameters in GD patients

We also studied the relationship between TNF-α, sTNF-R1, sTNF-R2 and related immune indicators (Table 5).

Table 3 Characteristics and serum TNF-α, sTNFRs levels in low FT3 GD group and high FT3 GD group.

Variables	Low FT3	High FT3	*P*
n	36	36	–
Age (year)	42 ± 11	40 ± 11	0.585
Sex (F/M)	24/12	28/8	0.293
Smoking (Y/N)	10/26	6/30	0.257
BMI (kg/m^2)	21.8 ± 2.80	21.2 ± 4.14	0.274
SBP (mmHg)	128 ± 17	133 ± 15	0.674
DBP (mmHg)	77 ± 9	77 ± 8	0.706
Duration (month)	2 (0.5–24)	1.5 (0.5–8.5)	0.793
FT3 (pmol/L)	9.66 (6.04–13.1)	30.6 (24.4–33.9)	**<0.001**
FT4 (pmol/L)	23.9 (13.2–36.8)	61.5 (54.9–69.0)	**<0.001**
TSH (mIu/L)	0.01 (0.01–0.02)	0.01 (0.01–0.02)	0.602
TRAb (IU/L)	5.14 (2.74–12.6)	12.0 (6.18–24.0)	**0.007**
TPOAb (IU/mL)	53.5 (2.40–765)	307.1 (47.3–1013)	0.055
TgAb (IU/mL)	1.20 (0.30–85.3)	8.40 (0.40–74.0)	0.187
Thyroid VolT (mL)	14.5 (9.37–17.5)	20.7 (14.1–30.8)	**0.002**
IgG (g/L)	14.5 ± 4.23	13.5 ± 3.39	0.406
IgM (g/L)	1.33 ± 0.59	1.00 ± 0.48	**0.046**
IgA (g/L)	2.05 (1.68–2.82)	1.92 (1.30–2.36)	0.254
C3 (g/L)	1.22 (1.01–1.43)	1.30 (1.16–1.53)	0.345
C4 (g/L)	0.32 (0.29–0.38)	0.37 (0.31–0.42)	0.231
CD3 (/μL)	1024 (767–1144)	1373 (1053–1640)	0.062
CD4 (/μL)	594 (407–720)	758 (605–880)	**0.047**
CD8 (/μL)	400 (319–570)	579 (341–638)	0.214
CD19 (/μL)	228 (192–312)	290 (149–402)	0.855
CD16 + 56 (/μL)	299 (187–360)	207 (160–285)	0.165
CD3 (%)	65.1 ± 6.68	69.3 ± 8.71	0.102
CD4 (%)	36.8 ± 5.35	39.4 ± 6.56	0.133
CD8 (%)	26.8 ± 5.39	27.9 ± 7.79	0.471
CD19 (%)	17.0 ± 5.78	16.1 ± 7.03	0.660
CD16 + 56 (%)	17.7 ± 6.05	14.4 ± 8.52	**0.048**
TNF-α (pg/mL)	26.2 (4.95–74.3)	48.7 (7.51–383)	0.150
sTNF-R1 (pg/mL)	26.1 (15.2–41.5)	44.7 (24.5–69.0)	**0.008**
sTNF-R2 (pg/mL)	19.2 (11.9–36.8)	29.3 (16.0–43.6)	0.126

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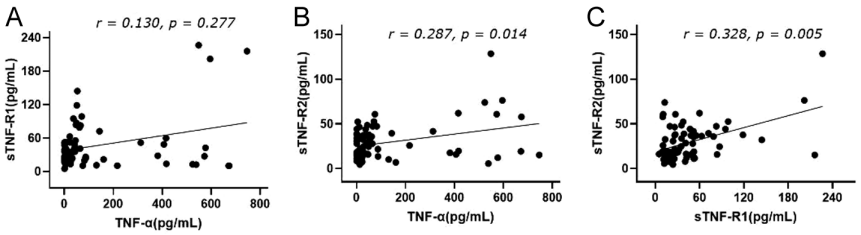

Figure 2
Correlations between serum levels of TNF-α and sTNF-R1 (A), TNF-α and sTNF-R2 (B), sTNF-R1 and sTNF-R2 (C) in GD group.

Relevant immune indicators such as complement C4 (C4) and the percentage of CD4, CD8, and CD19 showed differences between GD group and HC group (P=0.004 for C4 and CD19, P=0.001 for CD4 and P=0.002 for CD8, respectively, Table 1). However, absolute counts for CD3, CD8 and CD16+56 showed differences between the two groups (P=0.026 for CD3, P<0.001 for CD8 and P=0.001 for CD16+56, respectively, Table 1). Only serum levels of sTNF-R1 showed a significant positive correlation with C3 (P=0.045). No correlation was found between these immune parameters and TNF-α or sTNF-R2 (Table 5).

Multiple linear regression analysis of serum sTNF-R1 and sTNF-R2 in GD patients

To analyse whether sex, age, SBP, DBP, BMI, duration, thyroid volume, FT4, TSH, TRAb, TPOAb, TgAb, sTNF-R1 or sTNF-R2 had significant effects on FT3, we performed multiple linear regression analysis. As is shown in Table 6, the serum levels of sTNF-R1 (P<0.001) and FT4 (P=0.023) may play an important role in the serum level of FT3.

Discussion

Analysis of serum TNF-α level in GD patients

Investigation of the role of cytokines in the onset and course of autoimmune thyroid diseases has been ongoing for decades. A pro-inflammatory cytokine, TNF-α, is engaged in the induction and maintenance of immune responses. It is thought to play a critical role in autoimmune thyroid diseases, including Graves' disease.

As it has been reported that TNF-α is usually short-lived in the peripheral blood, differing changes in serum levels of TNF-α have been reported in patients with hyperthyroidism. Chopra *et al.* documented serum levels of TNF-α in hyperthyroidism and hypothyroidism for the first time (10). Consistent with our findings, they found no abnormality in serum levels of TNF-α with these diseases. Siddiqi *et al.* found that serum TNF-α was undetectable in both patients and controls as well (12). It has been argued that mononuclear cells critical to autoimmune thyroid disease may be different from those critical for the production of TNF-α (10). In contrast, Celik *et al.*

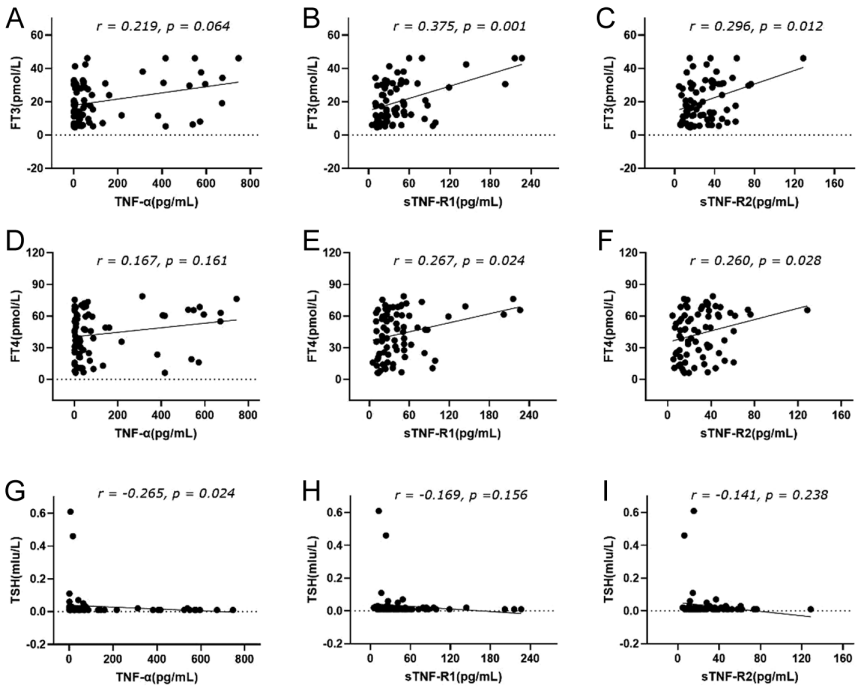

Figure 3
Correlations between serum levels of FT3 (A–C), FT4 (D–F), TSH (G–I) and TNF-α, sTNFRs in GD group.

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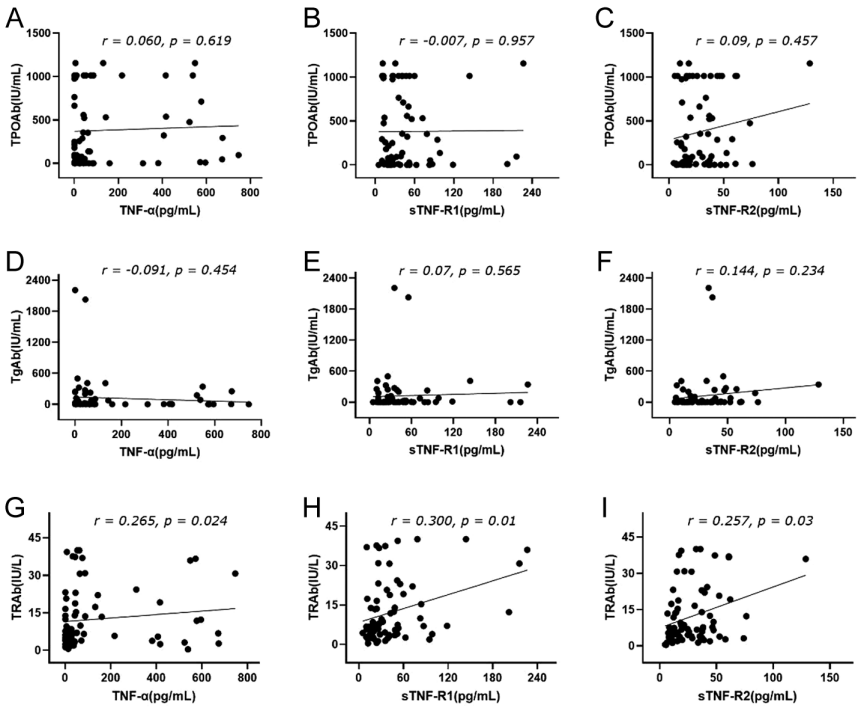

Figure 4
Correlations between serum levels of TPOAb (A–C), TgAb (D–F), TRAb (G–I) and TNF-α, sTNFRs in GD group.

reported high median serum TNF-α levels in Graves' disease patients, which could not revert to normal after 6 weeks of antithyroid medication. Elevated levels of TNF-α in the serum of GD patients may be affect to a systemic response to disease or to an intrathyroidal antigen–antibody reciprocity. Increase of cytokines in the thyroid gland may lead to the production of other cytokines by autoreactive cells, thus perpetuating the autoimmune reaction (13). From thyroid tissues of longstanding, relapsing Graves' disease patients, Paschke *et al*. could not detect any TNF-α and suggested that TNF-α may be distinctly lower due to the chronic phase of the disease (19). It has been argued that tissue levels are not reflected in serum concentrations (10, 20). Aysen *et al*. were surprised to find that serum TNF-α levels were slightly lower in patients with hyperthyroidism. All the patients were given antithyroid medication until they became euthyroid. After the treatment, TNF-α returned to normal levels, comparable with healthy controls (14). Similarly, a reduced TNF response after repeated stimulation has been demonstrated *in vitro* (7).

The results of this study showed that there was no significant difference in serum TNF-α between GD patients and healthy controls. Possible explanations are as follows: (1) Serum TNF-α may be less stable than the soluble receptors (21, 22). Its concentration varies at different stages of the disease. (2) TNF-α is produced at lower levels in affected tissues and degrades after its production. (3) TNF-α is a secretory product of fat tissue (23). The serum level of TNF-α in the GD group did not increase significantly, which may be related to the significant decline in BMI due to hyperthyroidism. (4) It is also acknowledged that Graves' disease is a common autoimmune disorder with a genetic predisposition. Owing to the biological effect of TNF-α on the thyroid gland and its gene location, TNF-α may be able to influence an individual's susceptibility to GD (24). Gu LQ reported that both the G alleles of TNF-alpha −238 and +419 SNPs conferred a higher risk of GD than did A alleles. No significant difference in −308 allelic frequency was observed. Recently, Tu Y reported that, after ethnicity stratification, significant genetic GD susceptibility was detected in the European population in all genetic models. In contrast, no significant association could be detected in the Asian population (24). TNF-α gene polymorphism may play an important role as well. The influence of gene polymorphism on the occurrence and development of GD deserves further in-depth study. (5) In this research, the people we recruited were all Chinese; different races and regions may lead to different results. The duration in patients varied from 1 week to 10 years. The discrepancies among reports may result from differences in methodology, patient selection and chronicity of the autoimmune disease.

It was interesting to find that TNF-α was significantly negatively correlated only with TSH. TSH is produced by the anterior pituitary, so it serves as a sensitive index reflecting the function of the hypothalamus–pituitary–thyroid axis.

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Table 4 Spearman's bivariate correlations between TNF-α, sTNFRs and thyroid indices in GD and HC groups.

	GD						HC					
	TNF-α		sTNF-R1		sTNF-R2		TNF-α		sTNF-R1		sTNF-R2	
Parameters	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
FT3 (pmol/L)	0.219	0.064	0.375	**0.001**	0.296	**0.012**	0.133	0.265	−0.010	0.936	0.063	0.599
FT4 (pmol/L)	0.167	0.161	0.267	**0.024**	0.260	**0.028**	0.097	0.417	−0.182	0.126	−0.190	0.109
TSH (mIu/L)	−0.265	**0.024**	−0.169	0.156	−0.141	0.238	−0.163	0.172	−0.017	0.890	0.205	0.084
TRAb (IU/L)	0.265	**0.024**	0.300	**0.010**	0.257	**0.030**	−0.441	0.235	−0.342	0.367	0.342	0.367
TPOAb (IU/mL)	0.060	0.619	−0.007	0.957	0.090	0.457	−0.004	0.981	0.203	0.181	0.189	0.214
TgAb (IU/mL)	−0.091	0.454	0.070	0.565	0.144	0.234	−0.783	**0.013**	−0.533	0.139	0.210	0.588

TSH is the most sensitive and valuable objective index reflecting thyroid function. However, the TSH levels of GD patients in this study were generally low and imprecise; approximately 92% of them were distributed at 0.01, 0.02 and 0.03 mIu/L. Further investigation is needed to confirm the possible relationships and related mechanisms. These controversial results further suggest that it is necessary to measure the levels of both types of sTNFRs to assess TNF-α levels as a whole.

Analysis of serum sTNF-R1 and sTNF-R2 levels in GD patients

Our findings were consistent with those of previous studies demonstrating elevated levels of sTNF-R1 and sTNF-R2 in GD patients. These two soluble receptors were significantly correlated with the parameters of thyroid function. As sTNFRs are induced by TNF-α, their elevated concentrations in serum may reflect the activity of TNF-α even if TNF-α itself is not detected to be significantly increased. Indeed, there is considerable evidence suggesting that sTNF-R1 and sTNF-R2 are more reliable markers of TNF-α and inflammatory activity (25).

Only a few studies have included the measurements of serum levels of soluble cytokine receptors such as sTNF-R1 (2, 26, 27, 28, 29) and sTNF-R2 (30). Reuter *et al*. showed a significant correlation between elevated sTNF-R1 levels and increased levels of FT4, FT3, TRAb and thyroidal Tc-99m uptake in 39 hyperthyroid patients (29). Similar findings were reported by Díez *et al*. who also found increased sTNF-R1 levels in parallel with increased TNF-α serum levels in 18 Graves' patients. However, they did not find any correlation between TNF-α or sTNF-R1 levels and FT4 and T3 levels in their hyperthyroid patients (28).

The level of FT3 is the most sensitive and valuable index reflecting thyroid function and is not affected by thyroglobulin. According to the median value of FT3, the GD patients were further divided into a high FT3 GD group and a low FT3 GD group. The serum levels of sTNF-R1 in patients in the high FT3 GD group were significantly greater than those in the low FT3 GD group, but there was no significant difference between TNF-α and sTNF-R2. Multiple linear regression analysis suggests that serum levels of sTNF-R1 and FT4 may play an important role in the serum level of FT3. FT4 is an important indicator in routine clinical diagnosis and can monitor the inhibition treatment of thyroid function. Our findings show that serum sTNF-R1 was significantly correlated with thyroid function, which is consistent with the interaction between different signal pathways of sTNF-R1 and sTNF-R2

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Table 5 Spearman's bivariate correlations between TNF-α, sTNFRs and immune indices in GD group.

Parameters	TNF-α		sTNF-R1		sTNF-R2	
	r	*P*	*r*	*P*	*r*	*P*
C3 (g/L)	−0.012	0.936	0.297	**0.045**	0.269	0.071
C4 (g/L)	0.069	0.650	0.203	0.175	0.290	0.051
CD3 (/μL)	0.145	0.353	0.128	0.415	0.226	0.145
CD4 (/μL)	0.097	0.534	0.096	0.540	0.187	0.229
CD8 (/μL)	0.227	0.143	0.109	0.488	0.195	0.210
CD19 (/μL)	0.194	0.214	−0.065	0.680	0.128	0.413
CD16 + 56 (/μL)	−0.177	0.255	−0.022	0.888	0.049	0.756
CD3 (%)	0.147	0.347	0.036	0.819	0.077	0.623
CD4 (%)	0.014	0.929	−0.007	0.965	0.033	0.834
CD8 (%)	0.219	0.158	0.021	0.892	0.109	0.485
CD19 (%)	0.127	0.418	−0.067	0.671	−0.032	0.840
CD16 + 56 (%)	−0.219	0.159	−0.051	0.745	−0.038	0.807

stimulated by TNF-α, as reported by Petrus (31). The main function of sTNF-R1 in the signal pathway is to promote inflammation and apoptosis, while the main function of sTNF-R2 is to promote survival. Therefore, sTNF-R1 may play a more important role in the development of GD, but the underlying mechanism needs to be further explored.

We also found that TRAb level was positively correlated with TNF-α, sTNF-R1 and sTNF-R2. TRAb is a specific biomarker for GD (32, 33). It can be used in the study of immunology and pathogenesis of GD. It plays an important role in the differential diagnosis between GD and other thyroid diseases. TNF-R2 is a member of the costimulatory TNFRs. TNF-R2 transduces signals through TNF receptor-associated factors (TRAFs). TRAFs are critical for activating the canonical NF-κB, noncanonical NF-κB, and MAPK pathways. Engagement of TNF with TNF-R1 leads to the release of silencer of death domain protein (SODD) and the formation of a receptor-proximal complex, including some important adaptor proteins. These adaptor proteins, in turn, recruit other key pathway-specific enzymes. These enzymes then become activated and initiate downstream events leading to apoptosis, NF-κB activation, and JNK activation (34). This results in an imbalance of humoural and cellular immune responses leading to the appearance of autoantibodies. sTNFRs may play an important role in the occurrence and development of Graves' disease.

Table 6 Multiple linear regression analysis of serum sTNF-R1 and sTNF-R2 levels in GD group.

Parameters	β	*t*	*P*	95% CI	
FT4 (pmol/L)	0.840	7.725	<**0.001**	0.385	0.666
sTNF-R1 (pg/mL)	0.186	2.424	**0.023**	0.008	0.105

Analysis of serum TNF-α, sTNFRs and immune indices in GD patients

A large number of studies show that the aetiology of GD is mainly on the basis of polygenetic inheritance; stress factors (such as mental stimulation) induce the occurrence of the autoimmune response. Antibodies are produced to bind with the TSH receptor, being the surface receptor of thyroid cells, stimulating thyroid cells to produce a high level of thyroid hormone, inducing immune disorder, resulting in infiltration by a large number of T lymphocytes, especially CD4+T-cells, causing an imbalance in the proportion of helper T-cells (Th cells) and regulatory T-cells (Treg cells) and an imbalance of Th1 and Th2 cells, leading to abnormal expression of many cytokines (35). Professor Zhang Yanyun found that, in GD patients, the number of important Treg cells decreased significantly, the polarisation of dendritic cells shifted, and the proportion of its subtype plasma-like dendritic cells increased significantly, which can cause the increase of IFN-α secretion, leading to Treg cell apoptosis and GD immune imbalance. The mechanism and key regulatory targets of immune imbalance in GD are revealed, which provides an important basis for new immune intervention and treatment strategies (36).

CD4+T-cells play an important role in selecting high-affinity B-lymphocytes to differentiate into memory cells or plasma cells and maintaining humoural immune response. As shown in this study, immune indices such as C4, CD4, CD8, CD19 and CD16+56 changed significantly in the patient group. The TNF system regulates immune function by inducing the expression of MHC class II molecules, stimulating the differentiation of monocyte/macrophage precursors, activating cells such as natural killer cells, and inducing the synthesis of cytokines. As a result, the autoimmune function of patients may

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be stimulated or aggravated and the composition of lymphocyte subsets may change. Compared to normal controls, C4 was significantly elevated in Graves' patients. However, only serum levels of sTNF-R1 showed a significant positive correlation with C3. This indicates that sTNF-R1 may have a closer relation with the autoimmune mechanisms of Graves' disease. The inflammatory mediators in hyperthyroid patients may induce C3 produced by hepatocytes, epithelial cells and fibroblasts (37). The reason for these contradictory observations may be the differential inducers of C3 and C4. It has been reported that the synthesis of C3 is dependent on IL-1, IL-2 and TNF-α, whereas the production of C4 is dependent on IL-6 and TNF-α cytokines (38). Accordingly, different cytokine profiles in hyperthyroid patients may lead to differences in C3 production. The detection of thyroid autoantibodies and lymphocyte subsets in GD patients may have practical significance for the guidance of clinical treatment, the evaluation of disease activity and the assessment of the prognosis of this disease.

Analysis of research limitations

It must be addressed that there are several limitations to our study. First, it was a cross-sectional design with small sample sizes. This study could not explain the cause–effect connection between increased serum sTNFRs and varying parameters of thyroid and immune function in Graves' disease patients. Second, our research was conducted with Chinese participants, and the generalisability of our results needs to be evaluated. Third, due to the small sample sizes, we did not further group the patients according to the course or severity of Graves' disease to study correlations with the factors we measured. Therefore, further studies should be conducted to validate the results of our research and handle these limitations.

In summary, our results suggest that Graves' disease may be associated with an inflammatory profile. However, the mechanism is still unclear. Additional studies need to be done to determine if increased sTNFRs reflect a state or trait marker and to determine the functional significance of altered peripheral cytokine levels.

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

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Author contribution statement
Q Z, J S and M T participated in the design of the study, data collection, analysis of the data, and drafting of the manuscript. D Z and X W conceived the study, participated in its design and revised the manuscript. Y G and M T participated in the analysis of the data and revised the manuscript. J S, X W, D Z and Y G participated in data collection. All authors read and approved the final manuscript.

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