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Clinical characteristics of thyroid eye disease and expression profile of peripheral blood immune cells

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This study included patients with thyroid eye disease (n = 81) and Graves' disease (n = 165) for a cross-sectional study. Then, peripheral blood mononuclear cells (PBMCs) were collected from patients with thyroid eye disease (n = 5), Graves' disease (n = 15), and normal individuals (n = 5) for RNA-seq. WGCNA analysis was conducted on the RNA-seq results to identify genes related to thyroid eye disease, and these genes were verified by RT-qPCR. The results suggest that: (1) Neutrophil counts were elevated in the TED group compared to the GD group. (2) Ten hub genes, MMP9, BPI, CD177, MPO, CEACAM8, CEACAM1, SLPI, AZU1, KCNJ15 and TNFRSF10C, were identified. The above hub genes were positively correlated with neutrophils.

Keywords Thyroid eye disease, Graves' disease, WGCNA (weighted gene co-expression network analysis), Neutrophil, RNA-seq

Thyroid eye disease (TED), alternatively referred to as Graves' Ophthalmopathy, is an autoimmune disease involving orbital tissue. Approximately 20–40% of Graves' Disease (GD) patients will develop TED[1]. The prevalence of TED in the population is about 9/10,000, with women having a higher prevalence than men[2]. Signs and symptoms of TED include dry eye and trachoma, photophobia, excessive lacrimation, diplopia, upper eyelid contracture, edema, periorbital tissue and conjunctival erythema, proptosis, and in about 3–5 percent of patients with TED, severe pain, corneal ulceration, or compressive optic neuropathy[3].

At present, the pathogenesis of TED is still under investigation. It is now mainly believed that cellular and humoral immunity play a key role in the pathogenesis of TED, with T lymphocytes interacting with orbital fibroblasts via the CD40 - CD154 pathway, and orbital fibroblasts being activated to produce large quantities of cytokines and extracellular matrix, which in turn leads to severe orbital inflammation and tissue remodelling[3,4]. Thyrotropin receptor (TSHR) is the main autoantigen of GD, and thyrotropin receptor antibody (TRAb) is a characteristic and pathogenic autoantibody against TSHR in GD patients. Some studies detected overexpressed TSHR mRNA in orbital connective tissue of TED patients, and it was hypothesised that TSHR expressed on orbital fibroblasts was the cross-transgressing antigen that induced autoimmune reactions in TED patients[5,6]. Orbital fibroblasts also express insulin-like growth factor 1 receptor (IGF-1R), and TSHR binds to IGF-1R to form physical and functional signalling complexes involved in TED pathogenesis[7].

The current treatment of choice for moderately severe patients is intravenous glucocorticoid, however, glucocorticoid therapy may bring adverse effects such as psychiatric symptoms, new-onset diabetes, and severe infections; therefore, in some cases where the adverse effects of glucocorticoid therapy are unacceptable, second-line treatments such as orbital radiation therapy, and immune-targeted therapies have been proposed (e.g., rituximab, cyclosporine, mycophenolatemofetil, methotrexate, teprotumumab, etc.)[8,9]. TED is a challenging disease that not only significantly reduces the quality of life of patients but also affects their psychological health, and no cure for TED has yet been found, so research into the immune mechanisms of TED is important for the diagnosis and treatment of the disease.

Gene co-expression network analysis with a weighted approach, known as WGCNA, is a systems biology technique that delineates the interconnected patterns of gene expression across various samples. Genes with highly correlated expression are divided into different modules. Highly correlated genes were clustered into different modules by WGCNA to explore the association between different modules and sample phenotypes, and to search for hub genes within the modules as potential biomarkers or therapeutic targets for subsequent

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Items	GD (n = 165)	TED (n = 81)	P value
Age (years)	41.00 (30.50, 50.00)	48.00 (32.50, 54.00)	< 0.01
Female (%)	59.40	46.90	0.064
FT3 (pmol/L)	23.03 (12.49, 32.62)	9.83 (5.33, 23.03)	< 0.01
FT4 (pmol/L)	56.70 (35.68, 100.00)	30.45 (16.55, 62.75)	< 0.01
TSH (mIU/ml)	0.005 (0.005, 0.005)	0.005 (0.005, 0.145)	< 0.01
TRAb (IU/mL)	13.21 (6.75, 29.67)	16.00 (4.78, 28.76)	0.462
TGAb (IU/mL)	104.70 (24.90, 535.60)	19.88 (11.67, 436.30)	< 0.01
TPOAb (IU/ml)	176.70 (47.94, 447.80)	59.42 (12.28, 273.75)	< 0.01

Table 1. Comparison of basic sex, age, and thyroid function between the GD group and TED group.

Items	GD (n = 165)	TED (n = 81)	P value
WBC($\times 10^9/L$)	6.30(5.34, 7.44)	6.80(5.50, 8.67)	0.026
M($\times 10^9/L$)	0.50(0.40, 0.63)	0.50(0.36, 0.60)	0.049
L($\times 10^9/L$)	2.10(1.63, 2.60)	2.20(1.80, 2.96)	0.188
N($\times 10^9/L$)	3.30(2.60, 4.13)	3.70(2.70, 5.14)	0.025
B($\times 10^9/L$)	0.00(0.00, 0.00)	0.00(0.00, 0.02)	< 0.01
E($\times 10^9/L$)	0.10(0.10, 0.20)	0.10(0.07, 0.20)	0.655

Table 2. Comparison of peripheral blood immune cell-related indices between the GD group and TED group.

analyses. In recent years, WGCNA has been widely used in the study of complex molecular mechanisms of various diseases, which helps to find out the therapeutic targets and diagnostic markers related to the diseases.

The development of TED may be associated with abnormal alterations in the function of various immune cells, including memory B cells, helper T lymphocytes, resting NK cells, M0-type macrophages, M1-type macrophages, resting dendritic cells, activated mast cells and neutrophils[10]. At present,the pathogenesis of TED is not completely clear, and its pathophysiological process is very complex, which is the result of many genes involved in regulation. In order to screen the gene modules and hub genes that play an essential function in the development of TED, the present study explored the risk factors for the development of TED starting from a retrospective clinical study, and further constructed gene expression profiles by eukaryotic referential transcriptome sequencing (RNA-seq) of peripheral blood mononuclear cells (PBMCs) from TED, GD and healthy control subjects. We used bioinformatics methods such as WGCNA to mine the immune mechanism of TED and screen the core genes associated with it, and finally RT-qPCR for target gene validation to reveal the potential immunological mechanism of TED and provide the basis for the diagnosis and treatment of TED.

Results
Cross-sectional study
Comparison of basic sex, age, and thyroid function between the GD and TED groups
Free Triiodothyronine(FT3), Free Thyroxine(FT4), Thyroglobulin antibody(TGAb), and Thyroid peroxidase anti- body (TPOAb) were elevated and TSH and age were decreased in the GD group compared to the TED group ($P < 0.01$), as shown in Table 1.

Comparison of peripheral blood immune cell-related indices between the GD and TED groups
White blood cell(WBC), Neutrophil(N) and Basophil cell(B) were significantly lower in the GD group compared to the TED group [6.30 (5.34, 7.44) vs 6.80 (5.50, 8.67)×10^9/L, P_{WBC} = 0.026; 3.30 (2.60, 4.13) vs 3.70 (2.70, 5.14),P_N = 0.025; 0.00 (0.00, 0.00) vs. 0.00 (0.00, 0.02), P_B < 0.001]. Monocyte (M) was elevated in the GD group compared to the TED group [0.50 (0.40, 0.63) vs 0.50 (0.36, 0.60)×10^9/L, P_M = 0.049], as shown in Table 2.

Expression profiling to identify core genes in thyroid eye disease and correlation with peripheral blood immune cells
Comparison of basic age and thyroid function between TED and GD groups
Five patients in the TED group and 15 patients in the GD group were included, comparing the age and thyroid function levels of the two groups, and there was no significant difference between the two groups in terms of age, FT3, FT4,TSH, and TRAb (as shown in Table 3).

Weighted gene co-expression network analysis
The gene sequencing dataset contained 25 samples including TED patients (n = 5), GD patients (n = 15) and healthy controls (n = 5), and the overall expression levels of the samples were obtained by FPKM analysis as shown in Fig. 1A. The 25 samples expression matrix data were analysed by sample hierarchical clustering, the abscissa represents the samples and the ordinate represents the genes, the clustering results are shown in Fig. 1B, there are no obvious outlier samples in the figure, so there is no need to remove the outlier samples. In order to construct

Items	TED (n = 5)	GD (n = 15)	P value
Age (years)	39.60 ± 13.92	36.00 ± 7.01	0.054
FT3 (pmol/L)	6.06 (2.99, 11.80)	9.77 (4.88, 50.00)	0.104
FT4 (pmol/L)	15.30 (6.92, 32.87)	28.86 (15.28, 100.00)	0.140
TSH (mIU/ml)	0.286 (0.009, 33.945)	0.006 (0.005, 1.400)	0.321
TRAb (IU/mL)	11.60 (8.64, 15.40)	32.60 (1.84, 39.20)	0.315

Table 3. Comparison of basic age and thyroid function between TED group and GD group.

an ideal scale-free network inline with the biological significance (the nodes are negatively correlated with the number of connections, and the few nodes with more connections and stronger correlation are the key genes to be screened), based on the WGCNA analysis package to screen the soft threshold, as shown in Fig. 1Ca,Cb, the screening selects the soft threshold, i.e., $\beta = 14$, at which time the fitting coefficient R^2 is 0.9, which is close to 1, and the average connectivity infinitely tends to 0. The co-expression network was meticulously assembled following the procedural steps of WGCNA analysis, which was followed by hierarchical clustering. Subsequently, genes were categorized into 42 distinct modules utilizing the dynamic tree cut algorithm, ensuring that each module contained no fewer than 30 genes as the minimum threshold (as show in Fig. 1Da). After obtaining the gene modules by WGCNA analysis, the gene modules were correlated with the samples by Pearson correlation analysis, according to the criteria of absolute value of module correlation ≥ 0.5 and $p < 0.05$, we finally obtained 2 modules (lightcyan module and thistle2 module) were significantly correlated with TED occurrence, and their correlation coefficients were 0.58 and 0.74 respectively (as shown in Fig. 1Db). According to the |MM|>0.70 and |GS|0.30 screening conditions, 82 genes were obtained from the lightcyan module (show in Fig. 1Ea), and 10 core genes were obtained by applying cytoscape to analyse the interactions of these genes (show in Fig. 1Eb), which were: MMP9, BPI, CD177, MPO, CEACAM8, CEACAM1, SLPI, AZU1, KCNJ15 and TNFRSF10C.

Immune cell type analysis
Compared with GD and normal patients, the TED group had an increase in peripheral blood neutrophils, while T cells CD8, NK cells resting, and macrophages M0 were reduced, as shown in Fig. 2.

Analysis of the correlation between hub genes and immune cells
The Pearson correlation coefficients between key genes and immune cells were calculated, and the immune cells with $p < 0.05$ and higher correlation coefficients were screened (show in Fig. 3). The expression level of MMP9 was positively correlated with neutrophils (r = 0.96, $P < 0.001$). The expression level of BPI was positively correlated with neutrophils (r = 0.86, $P < 0.001$) and activated mast cells (r = 0.58, $P < 0.05$). The expression level of CD177 was positively correlated with neutrophils (r = 0.95, $P < 0.001$). The expression level of MPO was positively correlated with neutrophils (r = 0.78, $P < 0.01$), activated mast cells (r = 0.63, $P < 0.05$), and resting memory CD4 cells (r = 0.73, $P < 0.01$). The expression level of CEACAM8 was positively correlated with neutrophils (r = 0.90, $P < 0.001$). The expression level of CEACAM1 was positively correlated with neutrophils (r = 0.91, $P < 0.001$). The expression level of SLPI was positively correlated with neutrophils (r = 0.91, $P < 0.001$). The expression level of AZU1 was positively correlated with neutrophils (r = 0.82, $P < 0.01$), activated mast cells (r = 0.66, $P < 0.05$), and resting memory CD4 cells (r = 0.65, $P < 0.05$). The expression level of KCNJ15 was positively correlated with neutrophils (r = 0.96, $P < 0.001$). The expression level of TNFRSF10C was positively correlated with neutrophils (r = 0.87, $P < 0.001$).

RT-qPCR results
A total of 19 patients were included (7 patients in the TED group and 12 patients in the GD group), and the difference in the relative expression of TNFRSF10C between the GD group and the TED group was statistically significant ($P < 0.05$), with TNFRSF10C in the TED group being higher than that in the GD control group [1.963 (0.508, 290.488) vs. 0.451 (0.559, 1.278), $P_{TNFRSF10C} = 0.022$]. The difference in relative expression of MPO and SLPI was not statistically significant, as shown in Table 4.

Discussion
The pathogenesis of TED is a complex interplay involving a multitude of immune cells and cytokines, encompassing various T-lymphocyte subpopulations and B-lymphocytes[11]. In the context of TED, deviations are noted across various immune cell types, including memory B cells, helper T cells, resting natural killer (NK) cells, as well as both M0 and M1 subtypes of macrophages, additionally, resting dendritic cells, activated mast cells, and neutrophils exhibit irregularities[10]. In the present study peripheral blood WBC, N and B levels were elevated in TED patients compared to GD patients. A study by Joanna Szydełko et al. also suggested that GD patients with TED had significantly higher WBC, neutrophil and neutrophil-to-lymphocyte ratio ($p < 0.05$) compared to GD patients without TED[12]. This underscores a potentially significant link between neutrophils and the development of TED.

Neutrophils are the main phagocytes in the circulation and are the first line of defence in the non-specific immune defence. These cells guide dendritic cells, monocytes, and lymphocytes, playing a pivotal role in determining the onset and sustenance of the immune response within organisms[13]. Furthermore, the inflammatory microenvironment allows neutrophils to acquire the characteristics of antigen-presenting cells to present antigens and induce the activation of specific immunity[14]. Neutrophil recruitment during infection
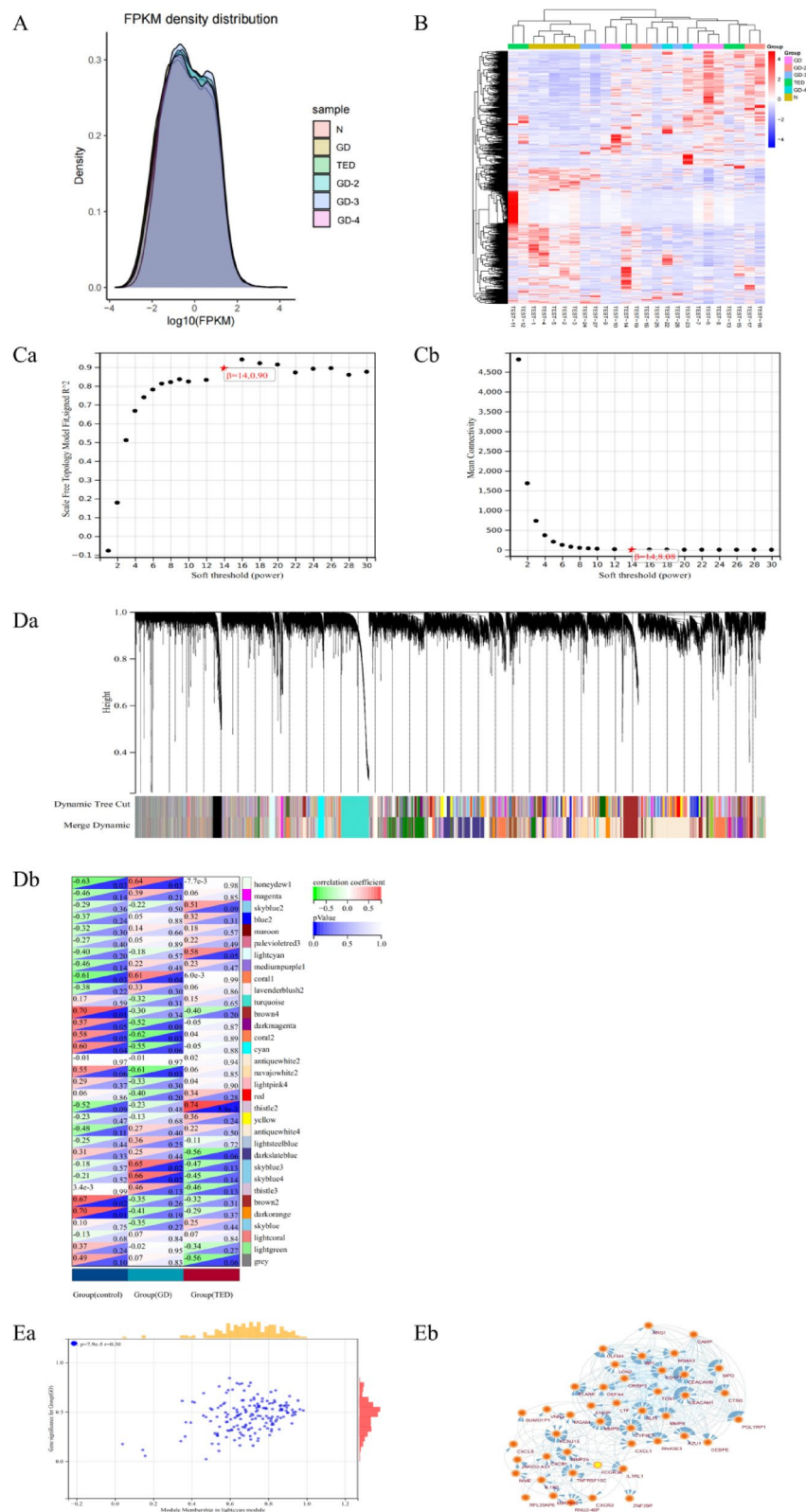
Fig. 1. Results of WGCNA. (**A**) Gene expression density map. (**B**) Sample clustering diagram. (**Ca**) The vertical coordinate represents the fitting coefficient R^2 value, and the horizontal coordinate represents the corresponding soft threshold. (**Cb**) The average connectivity value in vertical coordinates and the corresponding soft threshold in horizontal coordinates. (**Da**) The average connectivity value in vertical coordinates and the corresponding soft threshold in horizontal coordinates. (**Db**) Heatmap of the correlation between gene modules and TED. (**Ea**) Scatterplot of lightcyan module. (**Eb**) Lightcyan Module Gene Interaction Network Map.
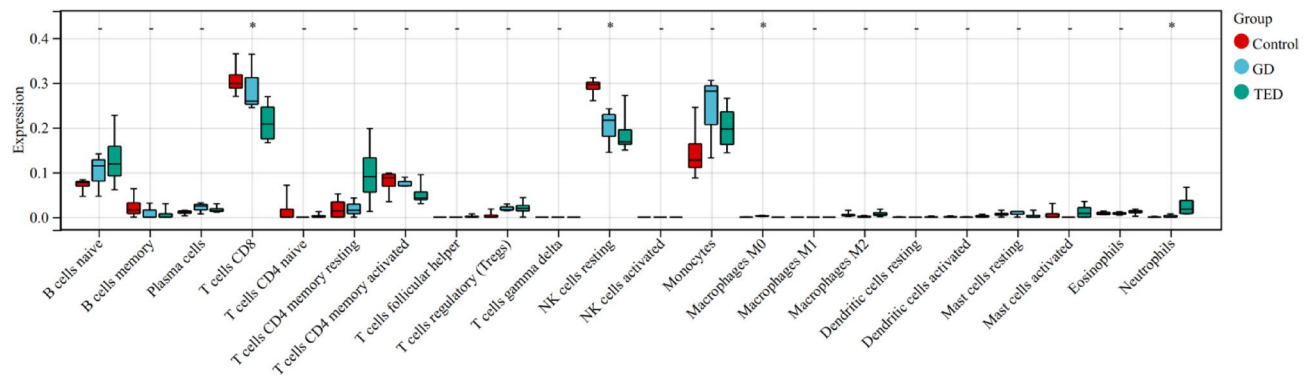
Fig. 2. Box plot of differential abundance of immune cells. (The vertical axis represents the expression of immune cells in each group, while the horizontal axis represents different types of immune cells. * indicates p value is less than 0.05).

may be necessary for protective immunity, but the neutrophil recruitment and inflammatory response that occurs during sterile inflammation may be deleterious. Aseptic inflammation is associated with a damage-associated molecular pattern (DAMP), which promotes the recruitment of neutrophils and macrophages and the production of pro-inflammatory cytokines and chemokines, and neutrophils release oxidants, proteases, and antimicrobial proteins, and prolonged aseptic inflammation can cause permanent damage to surrounding tissues[15,16]. More and more researchers are therefore looking at the role of neutrophils in autoimmune diseases.

Systemic autoimmune disorders are distinguished by the immune system's failure to differentiate between one's own tissues and foreign substances. Recent research has highlighted the role of neutrophil extracellular traps (NETs), which are pivotal in triggering and sustaining systemic autoimmune conditions, contributing to a complex pattern of inflammatory responses that can result in damage to various organs[17]. Taking SLE as an example, the level of pathogenic neutrophilsubset low-density granulocyte (LDG) in the blood of SLE patients is elevated, and LDG may lead to the pathogenesis of lupus and the development of end-organ damage through mechanisms such as enhancing pro-inflammatory response and synthesizing type I interferon, not only that, but LDG can easily form NETs, leading to excessive accumulation of NETs in the body[18,19]. The buildup of NETs in tissues has the capacity to trigger activation of B cells and plasmacytoid dendritic cells within the body,and this activation is mediated by Toll-like receptors and other intracellular sensors, which in turn, amplify inflammatory signaling cascades[20,21].

Advancements in high-throughput sequencing have introduced swift and potent bioinformatics techniques for the identifica- tion of key genes linked to the progression of diseases.

In our study, the lightcyan module and thistle2 module were significantly correlated with TED by WGCNA analyses. Ten hub genes were screened from the lightcyan module by gene interaction analysis: MMP9, BPI, CD177, MPO, CEACAM8, CEACAM1, SLPI, AZU1, KCNJ15 and TNFRSF10C, indicating that the occurrence and development of TED may be regulated by the above genes.

MMPs degrade most of the protein components in the extracellular matrix, disrupting the histological barrier to tumour cell invasion and playing a key role in tumour invasion and metastasis. For example, researchers have proposed that MMP- 9 could potentially be linked to the survival outcomes in various malignancies, including breast cancer, small cell lung cancer, and nasopharyngeal carcinoma[22–24]. MMP9 inhibitors reduce phosphorylation of SMAD2/3 in ribosomal protein S14 (RPS14)-deficient cells and increase erythropoiesis in the RPS14-deficient del(5q) MDS model through inhibition of the TGF-β pathway, supporting the potential for MMP9 inhibitors to be used clinically in the treatment of patients with the myelodysplastic syndrome del(5q)[25]. A study on GD showed that patients in the GD and TED groups had significantly higher serum concentrations of MMP-2 and MMP-9 than controls[26]. This suggests a potential association between the elevated levels of these matrix metalloproteinases and the disease pathology. Our experiment further validates the involvement of MMP-9 in TED occurrence through the gene level.

Neutrophils are a pivotal cellular constituent of the non-specific immune response, and their cytoplasmic particles contain a variety of antibacterial proteins and peptides, including BPI, which enhances bacterial phagocytosis and inhibits bacterial- induced inflammation by blocking the interaction of the LPS with the host pro-inflammatory pathway[27]. CD177, a distinctly human neutrophil adhesion molecule, experiences increased expression on the cellular surface within various inflammatory contexts. This molecule also functions as a specific binding escort for Platelet Endothelial Cell Adhesion Molecule-1 (PECAM- 1), playing a crucial role in cellular interactions[28]. PECAM-1/CD177 interaction favours neutrophil migration in an allele-specific manner[29]. Recent studies have shown that CD177 is associated with neutrophil activation during the duration of inflammation in ANCA-associated vasculitis[30]. MPO, also known as myeloperoxidase, is a haemoglobin protein synthesised during granulocyte differentiation and is a major component of neutrophils. Myeloperoxidase (MPO) generates potent oxidizing agents, including hypochlorous acid, which are instrumental in neutralizing and eliminating bacterial and other foreign pathogens. CEACAM8 and CEACAM1 are cell adhesion molecules associated with carcinoembryonic antigens. The primary function of CEACAM1 is to enable the transduction of signals from the extracellular environment through the cell membrane and into the intracellular space. Depending on whether it
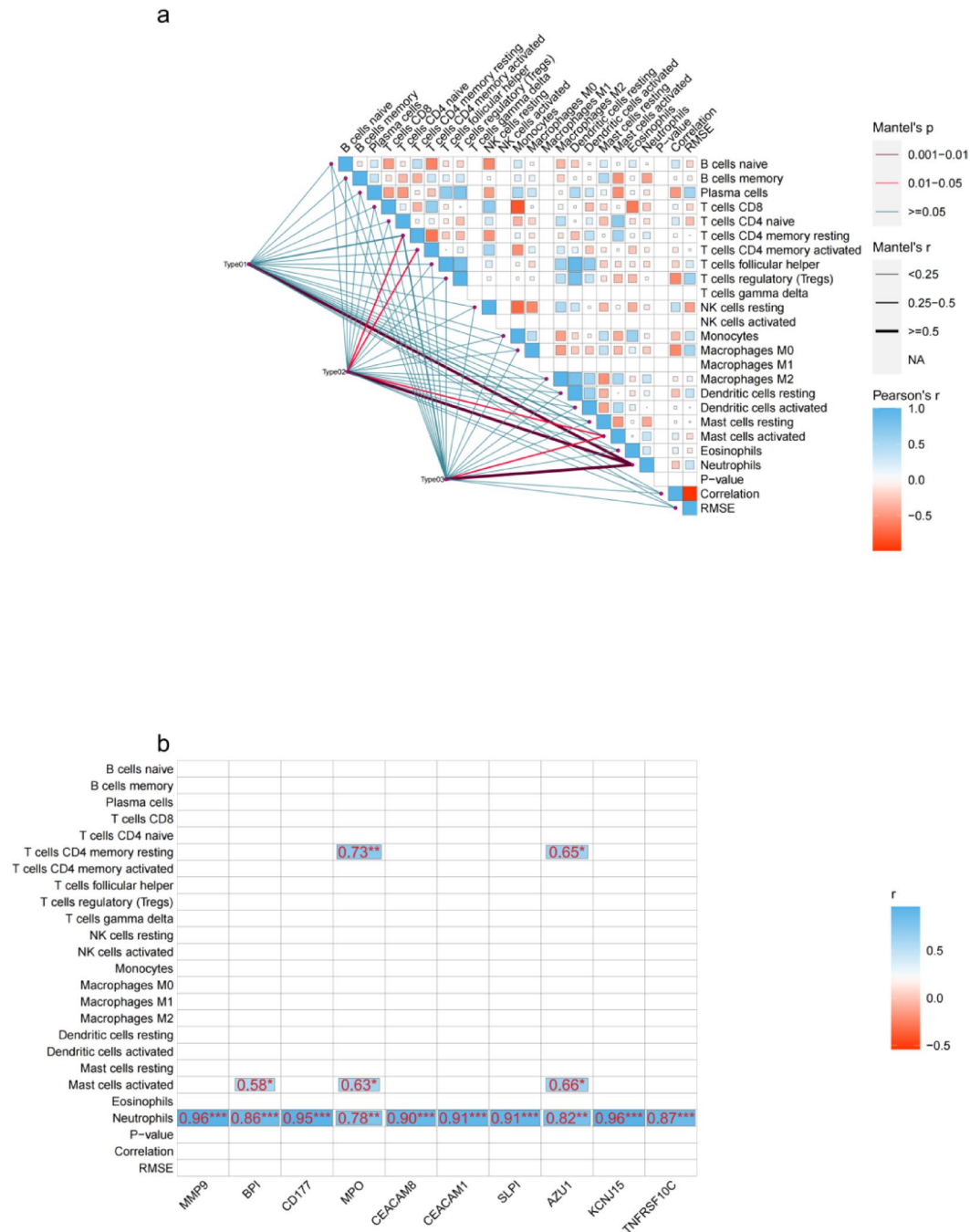
Fig. 3. Hub genes and immune cell relevance. (In figure a, Type01 contains MMP9, BPI, CD177. Type02 contains MPO, CEACAM8, CEACAM1. Type03 contains SLPI, AZU1, KCNJ15, TNFRSF10C. In figure b, the numbers represent the correlation coefficient between genes and immune cells, * indicates *p* value is less than 0.05, ** indicates *p* value is less than 0.01, *** indicates *p* value is less than0.001).

Relative expression of target gene	GD (n = 12)	TED (n = 7)	*P* value
TNFRSF10C	0.451 (0.559, 1.278)	1.963 (0.508, 290.488)	0.022
MPO	0.816 (0.404, 4.252)	1.290 (0.632, 38.903)	0.398
SLPI	0.995 (0.223, 5.937)	3.915 (1.206, 197.44)	0.091

Table 4. Comparison of the relative expression of target genes between GD group and TED group.

possesses a full-length or a truncated cytoplasmic tail resulting from alternative splicing, CEACAM1 can either propagate inhibitory signals or lack such inhibitory effects[31]. On human lung epithelial cells, CEACAM1 induces SHP-1 recruitment to CEACAM1 and TLR2 upon interaction with Catamonas, and SHP-1 negatively regulates TLR2-dependent activation of the PI(3)K-Akt-NF-kB signalling pathway to achieve immune escape[32]. Not only that, CEACAM1 is also considered to be an oncogenic factor, acting as an inhibitor of cell proliferation and a promoter of apoptosis in vivo[33]. CEACAM8 is considered a granulocyte or even polymorphonuclear neutrophil marker. SLPI plays a crucial role in preserving the integrity of barrier tissues by inhibiting tissue degradation and regulating the intensity of inflammatory reactions. It acts as a safeguard for homeostatic balance. Conversely, in the context of cancer, an elevated expression of SLPI within epithelial tumor cells could potentially augment their metastatic capabilities, thereby facilitating the spread of cancer[34]. The AZU1 gene encodes a preproprotein that undergoes proteolytic treatment to produce a mature aniline-loving cynophile granule antibiotic protein with monocyte chemotaxis and antimicrobial activity. The gene KCNJ15 encodes a protein that is an integral membrane protein and an inwardly rectifying potassium channel.

TNFRSF10C is an important member of the TNF receptor superfamily (TNFRSF). The protein encoded by this gene is an important immune regulatory receptor. TNFRSF10C is regarded as a major differential gene in the leukocyte transendothelial migration pathway. Studies have shown that it is associated with the prognosis of tongue cancer[35], triple-negative breast cancer[36], and colon cancer[37]. This indicates that TNFRSF10C may participate in the immune regulation of the tumor microenvironment by regulating the migration of immune cells. TNFRSF regulates the functions of immune effector cells by providing co-stimulatory signals[38]. Although the specific mechanism is not yet fully understood, the co-stimulatory effects of TNFRSF members (such as CD40, OX40, etc.) have been proven to be crucial for immune responses[39]. Studies of CD177, BPI,MPO, CEACAM8, CEACAM1, SLPI,AZU1, KCNJ15 and TNFRSF10C with TED have not yet been reported, whereas WGCNA analysis in the present experiment showed a potential association with the occurrence of TED.

Data from immune infiltration analyses indicated that the TED group exhibited a markedly higher expression level of neutrophils compared to both the GD group and the control group. This finding implies that there may be an important link between the presence of neutrophils and the development of TED. In order to explore whether there is an association between the core genes and the changes in neutrophils in the TED group, and to further carry out the study on the correlation between the immune cells and the core genes.The results showed that neutrophils were positively correlated with MMP9, BPI, CD177, MPO, CEACAM8, CEACAM1, SLPI,AZU1, KCNJ15, and TNFRSF10C. Suggesting that these ten hub genes are collectively involved in regulating neutrophil infiltration in TED populations to participate in the pathogenesis of TED, possibly amplifying the immune response or prolonging the duration of inflammation. The technique of RT-qPCR was employed to quantify the relative expression levels of the TNFRSF10C gene, a gene of central importance. The findings revealed notable disparities in the expression of TNFRSF10C between the GD group and the TED group. These results provide additional evidence supporting the role of TNFRSF10C in the pathogenesis of TED. These genes can be used as research targets for future TED-targeted therapies, and further studies and validation of their molecular signalling pathways and other mechanisms are needed.

Methods
Cross-sectional study
Subjects and enrolment criteria
A total of 246 patients individuals, aged between 18 and 85 years, who attended the Second Hospital of Fujian Medical University from September 2013 to September 2023 were collected. All participants were divided into TED group (81 cases) and GD group (165 cases). Enrolment criteria for patients in the GD group: 1.Between the ages of 18 and 85 years; 2.GD was diagnosed according to the diagnostic criteria of the Chinese Journal of Internal Medicine in 2007 "Guidelines for the Diagnosis and Treatment of Thyroid Diseases in China - Hyperthyroidism" (1. Clinical signs and symptoms of hyperthyroidism; 2. Diffuse thyroid enlargement, a few cases may not have goiter; 3. Decreased serum TSH concentration and increased concentration of thyroid hormones; 4. Protrusion of the eyeballs and other infiltrative ocular signs; 5. Pretibial mucous oedema; 6. TRAb or TSAb positivity. Of the above criteria, items 1-3 are necessary for diagnosis, and items 4-6 are auxiliary conditions for diagnosis). Enrolment criteria for patients in the TED group: 1.Between the ages of 18 and 85 years; 2.Meets diagnostic criteria for TED[40] (In the presence of eyelid recession, the diagnosis can be made with the combination of one of the following signs or evidence on examination: 1. Hyperthyroidism; 2. Eyeball protrusion, whose eyeball protrusion is ≥18mm, with a bilateral difference in eyeball protrusion of >2mm; 3. Involvement of extraocular muscles, limitation of eye movement; 4. Optic nerve dysfunction, including decreased visual acuity, abnormal pupillary reflexes, colour vision, and visual fields that cannot be explained by other pathologies. Patients lacking eyelid regression must have one of the following signs in addition to thyroid function abnormalities: extraocular muscle involvement, proptosis or optic nerve dysfunction, and exclude similar signs due to other eye diseases.) Exclusion criteria for case groups: 1.Combined malignant tumours, severe liver and kidney disease, pregnancy, severe infections, other autoimmune diseases, etc; 2.Taking antibiotics, lipid regulating drugs, immunosuppressants, glucocorticoids and other drugs that affect immune cells, lymphocytes, blood lipids, etc.

Statistical methods
All the data in this study were first collated using Office forms and then statistically analysed using the statistical package SPSS (v26.0). The Shapiro-Wilk test is employed to assess the normality of the variable distributions. In cases where continuous variables exhibit a normal distribution, they are expressed as the mean accompanied by the standard deviation, denoted as x ±s. For continuous variables that do not follow a regular distribution pattern, they are characterized by the median along with the interquartile range. Categorical variables, on the other hand, are represented by their respective rates. For comparisons between the two groups of data, when data

Gene	Primer sequences
MPO	FOR: GATGTGCAACAACAGACGCA REV: GAAGCCGTCCTCATACTCCG
SLPI	FOR: ACCCCAAACCCAACAAGGAG REV: ACGCAGGATTTCCCACACAT
TNFRSF10C	FOR: GGAACCATACCATGGCCCG REV: AGTAAGCTAGGACTGGCAGC
GAPDH	FOR: TGCCACTCCTCCACCTTTG REV: CGAACCACCCTGTTGCTGT

Table 5. Primer sequences for core genes and internal references.

adhered to a normal distribution, a t-test was utilized. For data that deviated from normality, a non-parametric test was applied. In instances of binary data, a chi-square test was the method of choice. All tests were bilateral, and a *P*-value of less than 0.05 was considered to denote statistical significance.

Expression profiling to identify core genes in thyroid eye disease and correlation with peripheral blood immune cells

Eukaryotic sibling transcriptome sequencing (RNA-seq)
Inclusion criteria for GD, TED and healthy controls were the same as in Part 1. Peripheral blood of GD patients (n = 15),TED patients (n = 5) and healthy controls (n = 5) attending the Second Affiliated Hospital of Fujian Medical University from February 2023 to June 2023 was collected, and PBMC cells were extracted and subjected to RNA-seq(Completed by Applied Protein Technology (Zhejiang) Co.,Ltd.). The process followed in this study was in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University (approval no. ECSAH of FMU [2024]546). Written informed consent was provided to all participants for this trial.

Construction of gene co-expression networks
The module eigengene (ME) was used as the main component to screen for potential correlation between genes and clinical traits, and the expression pattern of each module gene was summarised. Utilize phenotypic data and modules to assess the importance of genetic and clinical data and examine the relationship between models and modules. Candidate hub genes are selected from the gene pool with the highest degree of connectivity between modules. Critical modules were pinpointed through the computation of Gene Significance (GS) and Module Membership (MM) metrics. Absolute GS values tend to be greater in biologically important genes. In this study, criteria (GS > 0.30 and MM > 0.70 absolute values) were used to screen for potential hub genes.

CIBERSORT Immune cell type analysis
Immune cell type analysis was performed using the CIBERSORT algorithm to calculate the relative content (i.e., expression abundance) of each immune cell type in the samples, and comparative analyses were performed on the TED group, the GD group, and the normal control group to find whether there were any differences in immune cell types and content among the three groups.

Analysis of hub genes associated with immune cells
The linear correlation between the two random variables, Hub gene and immune cells, was determined by Pearson's correlation coefficient.

Experimental validation of fluorescence quantitative PCR
Samples were collected from PBMC extracted from peripheral blood cells of outpatient TED patients (n = 7) and GD patients (n = 12) attending the Second Affiliated Hospital of Fujian Medical University from November 2023 to January 2024, and total RNA was extracted from PBMC by the Trizol method, and the RNA was reverse transcribed into cDNA (TaKaRa). Real time PCR reaction of cDNA (TaKaRa) with upstream and downstream primers of internal reference gene and target gene, three replicate wells were used for each sample, and the CT (cycle threshold) value was determined by averaging the results from three replicate wells. This CT value indicates the number of cycles needed for the amplification of the template to reach a specific copy number threshold. The higher the CT value, the smaller the amount of the initial template involved in the reaction; conversely, the lower the CT value, the greater the amount of the initial template. The experimental data were analysed by $2^{-\Delta\Delta CT}$ to analyse the relative gene expression differences, using GAPDH as the internal reference, $\Delta CT = CT$ value of the target gene - CT value of the internal reference gene, and calculating the $2^{-\Delta\Delta CT}$ of the target genes in the case group and the control group, respectively.

Primer design
The sequences of the core genes MPO, SLPI, TNFRSF10C and the internal reference gene GAPDH were found on the NCBI web site and primers were designed to obtain the forward primer (FOR) and the reverse primer (REV) free of charge on NCBI, as shown in Table 5.

Statistical analysis
Comparison of non-normally distributed data was carried out using non-parametric tests and Pearson correlation analysis method was applied for correlation analysis. The fluorescence quantification results were applied to calculate the gene expression changes between different samples by the $2^{-\Delta\Delta CT}$ method. SPSS (v26.0), Sangerbox (v3.0) and Cytoscape (v3.10.3) software were used to analyse the data. *P*-value threshold of 0.05 is

used to determine statistical significance; values below this threshold suggest that the observed discrepancy is statistically noteworthy.

Data availability
The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

Accession codes
The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

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Declarations

Competing interest

The authors declare no competing interests.

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