“Immunohistochemistry study in human orbital tissue of thyroid eye disease”

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Abstract

Thyroid eye disease (TED) is an orbital inflammatory disease. The current management is suboptimal because of unclear mechanisms. Many *in vitro* studies have confirmed that the pathogenesis involves several pathogenic pathways and a network of infiltrating mononuclear cells, cytokines, and chemokines in the orbit. However, the *in vivo* environment is sometimes different from the *in vitro* situation. Orbital tissue is the best sample to reflect local inflammation. Additionally, immunohistochemistry (IHC) analysis of orbital tissue can reveal the local orbital immunity and correlate the microscopic changes with the macroscopic clinical features. Several IHC studies have been performed in TED but with several limitations, and the pathogenesis of some molecules remains unclear. Uncovering the molecular mechanism that plays a significant role in TED may help develop novel therapeutic strategies and optimize the management of TED.

In this study, we carried out staining of a large number of orbital tissue samples from patients with well-documented demographic and clinical data using antibodies against 18 proteins involved in multiple pathways that contribute to the pathogenesis of TED, and then investigated the primary mechanism. This study found that TSHR and IGF-1R signaling played a vital role in the pathogenesis of TED by activating pathways that resulted in the enhanced activation of orbital fibroblasts. T cells (CD3) and macrophages (CD68) were the main infiltrating immunocytes in the inflammatory stage of TED. IL-17A stimulated the differentiation of orbital fibroblasts into pre-adipocytes and myofibroblasts, and IL-17A, IL-23A, and IL-6 stimulated orbital fibroblasts to secrete hydrophilic hyaluronan and glycosaminoglycans. Furthermore, RANTES attracted T cells to migrate into the orbit, and MCP-1 promoted monocyte/macrophage migration into orbit. BAFF might participate in the primary mechanism by promoting B cell survival and maturation. The CD40-CD40L pathway is involved in the inflammatory response by mediating the interaction between orbital fibroblasts and T cells.

Targeting these key players may provide more effective and disease-specific treatment strategies.

Zusammenfassung

Die Schilddrüsenaugenerkrankung (TED) ist eine entzündliche Erkrankung der Augenhöhle. Die derzeitige Behandlung ist suboptimal, da die Mechanismen unklar sind. Viele In-vitro-Studien haben bestätigt, dass an der Pathogenese mehrere Pathogenitätswege und ein Netzwerk aus infiltrierenden mononukleären Zellen, Zytokinen und Chemokinen in der Orbita beteiligt sind. Die in vivo-Umgebung unterscheidet sich jedoch manchmal von der in vitro-Situation. Das Orbitalgewebe ist die beste Probe, um lokale Entzündungen zu erfassen. Darüber hinaus kann eine immunhistochemische (IHC) Analyse vom Orbitalgewebe die lokale orbitale Immunität aufzeigen und die mikroskopischen Veränderungen mit den makroskopischen klinischen Merkmalen korrelieren. Es wurden bereits mehrere IHC-Studien bei TED durchgeführt, allerdings mit einigen Einschränkungen, und die Pathogenese einiger Moleküle bleibt unklar. Die Aufdeckung der molekularen Mechanismen, die bei der TED eine wichtige Rolle spielen, könnte dazu beitragen, neue therapeutische Strategien zu entwickeln und die Behandlung der TED zu optimieren.

In dieser Studie haben wir eine große Anzahl von orbitalen Gewebeproben von Patienten mit gut dokumentierten demografischen und klinischen Daten mit Antikörpern gegen 18 Proteine angefärbt, die an mehreren Signalwegen beteiligt sind, die zur Pathogenese der TED beitragen, und anschließend den primären Mechanismus untersucht. Diese Studie ergab, dass die TSHR- und IGF-1R-Signalübertragung eine entscheidende Rolle bei der Entstehung von TED spielt, indem sie Signalwege aktiviert, die zu einer verstärkten Aktivierung der orbitalen Fibroblasten führen. T-Zellen (CD3) und Makrophagen (CD68) waren die wichtigsten infiltrierenden Immunzellen in der Entzündungsphase der TED. IL-17A stimulierte die Differenzierung der orbitalen Fibroblasten in Präadipozyten und Myofibroblasten, und IL-17A, IL-23A und IL-6 regten die orbitalen Fibroblasten zur Sekretion von hydrophilem Hyaluronan und Glykosaminoglykanen an. Darüber hinaus lockte RANTES T-Zellen an, in die Orbita einzuwandern, und MCP-1 förderte die Migration von Monozyten/Makrophagen in die Orbita. BAFF könnte an dem primären Mechanismus beteiligt sein, indem es das Überleben und die Reifung von B-Zellen fördert. Der CD40-CD40L-Signalweg ist an der Entzündungsreaktion beteiligt, indem er die Interaktion zwischen orbitalen Fibroblasten und T-Zellen vermittelt.

Die gezielte Beeinflussung dieser Schlüsselakteure könnte zu wirksameren und krankheitsspezifischen Behandlungsstrategien führen.

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List of abbreviations

ABC	Avidin-biotin complex
BAFF	B cell-activating factor
CAS	Clinical activity score
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD20	Cluster of differentiation 20
CD40	Cluster of differentiation 40
CD40L	Cluster of differentiation 40 Ligand
CD68	Cluster of differentiation 68
CD90	Cluster of differentiation 90
DAB	Diaminobenzidine
DON	Dysthyroid optic neuropathy
EUGOGO	European Group on Graves' Orbitopathy
FDA	Food and Drug Administration
FFPE	Formalin-fixed, paraffin-embedded
GD	Graves' disease
IFN-γ	Interferon gamma
IGF-1R	Insulin-like growth factor 1 receptor
IHC	Immunohistochemistry
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-16	Interleukin-16
IL-17A	Interleukin-17A
IL-23A	Interleukin-23A
JGU	Johannes Gutenberg University
K1-70	TSHR blocking monoclonal antibodies
LSAB	Labelled SA-biotin
MCP-1	Monocyte chemoattractant protein 1
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear Factor Kappa B

OAT	Orbital adipose tissue
OCT	Orbital connective tissue
OFs	Orbital fibroblasts
PBS	Phosphate-buffered saline
RCTs	Random clinical trials
RANTES	Regulated on activation, normal T cell expressed and secreted
SPSS	Statistical Package for the Social Sciences
TED	Thyroid eye disease
TGF-β	Transforming growth factor-beta
Th1	Type 1 helper T cells (Th1)
Th2	Type 2 helper T cells
Th17	Type 17 helper T cells
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSHR	Thyrotropin receptor

1 Introduction

Thyroid eye disease (TED), also termed Graves' ophthalmopathy, Graves' orbitopathy, or thyroid-associated ophthalmopathy, is an orbital inflammatory disorder related to autoimmune thyroid disease and the most common extrathyroidal manifestation of Graves' disease (GD) (1-6). GD is an autoimmune disorder caused by thyroid-stimulating hormone receptor (TSHR) antibodies (7), affecting ~2% of women and 0.2% of men worldwide (8). Ninety percent of TED is related to GD, and 10% of cases are related to hypothyroidism or a subclinical form of thyroid autoimmunity (euthyroid) (9).

The risk factors of TED are associated with thyroid dysfunction, smoking, female gender, genetics, and age (10-12). The signs and symptoms of TED are characterized by upper eyelid retraction, proptosis, double vision, chemosis, and rarely dysthyroid optic neuropathy (DON) (13). These abnormal clinical characteristics harm physical health, affect mental health, and pose great indirect and direct costs (14-17). The management, including medication, radiotherapy, and surgery, is suboptimal because of the unclear mechanisms underlying TED. With a better understanding of the pathogenesis of TED, several novel targeted therapies are undergoing random clinical trials (RCTs), and some have provided unexpected benefits for patients (18-24). However, as several pathways contribute to TED, these novel therapies still have limitations.

The disease process of TED involves underlying orbital inflammation and edema, orbital tissue adipogenesis and expansion, extraocular muscle enlargement, tissue remodeling, and fibrosis (3,25). TSHR and insulin-like growth factor-1 (IGF-1) receptor (IGF-1R) have been considered two crucial autoantigens in TED (3,5,26). T cell immunity is a major mechanism in TED (27-29). Orbital fibroblasts (OFs), which undergo proliferation and differentiation, have been considered target-effective cells in TED (26,30,31). The orbital tissue is infiltrated by T cells, B cells, and fibrocytes, and these cells produce different cytokines depending on the molecular signals they encounter in the microenvironment (2). These cytokines activate OFs to proliferate and differentiate (2). Activated OFs further differentiate into adipocytes and myofibroblasts, leading to adipogenesis and fibrosis, respectively. OF-mediated interactions with immune cells through the CD40-CD40L pathway result in the production of numerous cytokines and chemokines to maintain local inflammation

(25,27,32,33). Thus, the mechanism of TED involves several pathogenic pathways and a network of infiltrating mononuclear cells, cytokines, and chemokines in the orbit. Uncovering the molecular mechanism that plays a major role in TED may provide more evidence for developing novel therapeutic strategies and optimizing the management of TED.

1.1 Epidemiology and risk factors

1.1.1 Epidemiology

The exact overall incidence and prevalence of TED are difficult to summarize because the epidemiology varies with ethnicity, geographical location, and classification criteria. A well-known study in the United States showed that the incidence of TED in the local population is 2.9 per 100,000 males per year and 16 per 100,000 females per year (34). In Denmark, a 17-year study in the Danish population reported that the incidence of moderate-to-severe TED is 2.67 per 100,000 females per year and 0.54 for males (35). In Europe, the estimated overall prevalence of TED is 89.7/100,000 population, whereas that for mild, moderated-to-severe, and sight-threatening TED is 58.3/100,000, 29.6/100,000, and 1.8/100,000, respectively (36). The prevalence of TED in patients with thyroid dysfunction, especially those with GD, is usually higher than in the non-dysthyroid population. A meta-analysis and systematic review summarizing 57 studies in 26,804 subjects worldwide found that the overall prevalence of TED in GD is 40%, and the prevalence in Europe, Asia, North America, and Oceania is 38%, 44%, 27%, and 58%, respectively (37).

1.1.2 Risk factors

The risk factors for TED can be classified as endogenous and exogenous. Endogenous factors, which cannot be changed, consist of gender, age, genetics, and ethnicity. Exogenous factors, which are modifiable, include smoking, thyroid dysfunction, and radioiodine treatment (10). TED is more common in women than men (10,38,39), and the ratio (women: men) ranges from 3.4:1 to 4.2:1 according to different studies (10). However, men have been considered to progress more severely than women (40). The peak age is 40–69 years, and older patients, especially men, develop severe disease (10,41). Human leukocyte antigen, cytotoxic T-lymphocyte

antigen-4, and *TSHR* genes have been confirmed to be related to GD, but TED-associated genes remain unclear (11). A study reported that interleukin (*IL)-23*, *IL-1*, and *IL-1RA* genes might be involved in the development of TED (42). In a previous study, the prevalence of TED in Caucasians and Asians with GD was 42% and 7.7%, respectively (43). However, a recent meta-analysis found that the prevalence of TED in Caucasians and Asians with GD was 37% and 45%, respectively (37). The relevance of ethnic factors in TED remains controversial, possibly because of the different classification criteria applied in these studies.

For exogenous factors, smoking is the most concerning factor. Smoking accelerates the progression of TED and aggravates the severity of the disease (10-12,39,44). The risk of GD developing into TED is 2.75 times higher in smokers than in non-smokers (39). The number of daily cigarettes is also related to the severity of TED (11,12). Thyroid dysfunction is another concerning risk factor in TED, and both hyperthyroidism and hypothyroidism have been considered to promote the development and exacerbation of TED (11). Radioiodine therapy is an effective treatment for GD. However, it leads to the progression or development of TED in 15% to 39% of patients (11,42), especially in those with a smoking history, thyroid dysfunction, and high titer of TSHR antibodies (41). In addition, asymmetry is considered a symptom that implies more severe and active TED (45), and high cholesterol may exacerbate the disease (46).

1.2 Clinical features and classification

1.2.1 Clinical manifestations

The common clinical features are caused by inflammation, orbital fat tissue expansion, extraocular muscle enlargement, and the limitation of orbital bone (26,47). Initially, most patients have visible signs in orbital tissues, such as redness in the eyes, swelling in the lids, and bags under the eyes (13). Patients usually have gritty ocular sensation, overproduction of tears, fear of light, feeling of fullness in the eyelids, and a pressure sensation behind the eyes (3,13). As the disease progresses, additional and more severe clinical signs and symptoms develop. Eyelid retraction is the most common sign in TED (2,13,48,49) and is usually accompanied by the Kocher sign (13). Proptosis (protrusion of the eyeball) is also a prevalent symptom in TED and is involved

in lower lid retraction (13,38). Double vision (diplopia) occurs in the situation of tired or extremes of gaze (50). A recent meta-analysis and systematic review, which included 26,804 subjects, showed that the pooled prevalence of lid retraction, proptosis, and double vision in TED was 57%, 57%, and 36%, respectively (37). DON is a rare but severe complication in TED with an incidence of 5%–8% (51). More than 90% of cases result from nerve compression by enlarged extraocular muscles, and 5% is related to stretch optic neuropathy (51). In addition, corneal ulceration, superior limbic keratoconjunctivitis, and episodes of globe subluxation occur in some cases. Fortunately, these severe signs are rare (13).

1.2.2 Natural history

The natural course of TED involves several periods, and most patients with mild cases recover spontaneously. During the early stage of the disease, the clinical features progressively worsen and usually present as inflammatory signs and symptoms, and this phase is termed the active progressive stage. After the inflammation subsides, there is a plateau stage, and the symptoms and signs stop progressing and improve spontaneously. Finally, a static phase appears after the regression of inflammation, but fibrosis has developed, and the tissue cannot reverse to the original state and usually requires rehabilitative surgery. The above disease course follows a pattern known as Rundle's Curve (10,44,52-54). Patients treated with immunomodulatory therapies generally respond well in the active and inflammation phases but poorly in the inactive stage (55,56). Inactive patients require rehabilitative surgery, and emergency decompression surgery is usually performed in severe cases, such as TED complicated with DON (57,58). Therefore, assessing the activity and severity of TED is essential to distinguish patients and optimize the management.

1.2.3 Classification

The clinical activity score (CAS) is widely used to assess the activity status of TED. The assessment includes seven items: (1) spontaneous retrobulbar pain, (2) pain on attempted upward or downward gaze, (3) eyelid redness, (4) redness of conjunctiva, (5) swelling of caruncle or plica, (6) swelling of eyelids, and (7) swelling of the conjunctiva. One point is given to each item, and CAS ≥3 indicates the active phase, whereas CAS <3 indicates inactive TED (1,13,59,60). The assessment of TED severity

by the European Group on Graves' Orbitopathy (EUGOGO) depends on the degree of lid retraction, soft-tissue involvement, proptosis, diplopia, and sight-threatening. Patients can be classified as mild, moderate-to-severe, and very severe (sight-threatening) (1,13,59). Another severity assessment of TED is the NOSPECS classification, which includes no signs or symptoms (N), only signs but no symptoms (O), soft tissue involvement (S), proptosis (P), extraocular muscle involvement (E), corneal involvement (C), and sight loss (S) (1,59). In addition, the vision, inflammation, strabismus, and appearance (VISA) classification was developed by Dolman and Rootman and is widely used in Canada and the United States (61).

1.3 Diagnosis and management

1.3.1 Diagnosis

The diagnosis of TED usually depends on the clinical features of the eye, existence of autoimmune thyroid disease, and exclusion of other conditions included in the differential diagnosis (44,62). Eyelid retraction, eyelid swelling, proptosis, eyeball motility restriction (diplopia), corneal stippling or ulceration, and decreased visual acuity help make the diagnosis of TED (62). The diagnostic criteria for TED by Bartley is widely accepted (63). According to this criterion, TED should be considered if eyelid retraction is related to objective evidence of thyroid dysfunction, proptosis, optic nerve dysfunction, or restrictive extraocular myopathy. If eyelid retraction is absent, the diagnosis of TED needs to depend on proptosis, optic nerve dysfunction, or extraocular muscle involvement related to thyroid dysfunction and exclude the differential diagnosis simultaneously (63).

1.3.2 General management

The management varies with the heterogeneity of TED and the activity (active/inactive) and severity (mild/moderate-to-severe/very severe) of the disease. All patients with TED are required to quit smoking and maintain normal thyroid function (1,59,64). Local treatment, including artificial tears, ophthalmic gels, lubricant ointments, topical cyclosporine, and botulinum toxin A, can be used based on the eye symptoms of patients (65). The inactive/stable phase of TED is usually treated with rehabilitative surgery based on the severity of the disease, including decompression, blepharoplasty,

eyelid surgery, and strabismus surgery (58). Sight-threatening (very severe) TED usually requires emergency orbital decompression when patients show a poor response to medical treatment (57). For mild TED, because more than half of cases improve spontaneously without active treatment, the “wait-and-see” strategy is used for patients worried about the side effects of therapy (66). In addition, the antioxidant agent selenium delays the progression of the disease, decreases eye involvement, and improves the quality of life for patients with TED (67). Although a high selenium level is considered a risk factor associated with induced type 2 diabetes (68,69), a low dose of daily sodium selenite (200 μg [91.2 μg selenium]) for 6 months is recommended in mild TED (1,67).

1.3.3 Current management for moderate-to-severe/active TED

To date, corticosteroids have remained the first-line drug for moderate-to-severe and active TED (1,4,59,60,70-72). The most widely used corticosteroid regimen is a cumulative dose of 4.5 g intravenous methylprednisolone administered within 12 weeks (0.5 g/week, then 0.25 g/week, 6 weeks each) (1,59,60,73). Unfortunately, the response rate to corticosteroids is 50%–80% and is heavily dependent on the stage of disease and the dose of corticosteroids (1,72). Although high doses of corticosteroids improve the effectiveness of treatment, increased doses are associated with a higher rate of side effects (74). The morbidity and mortality of intravenous corticosteroid injections are 6.5 and 0.6%, respectively (75). Furthermore, corticosteroids temporarily relieve inflammation and edema by suppressing the function of T cells, B cells, macrophages, and immunocompetent cells (74) but do not prevent tissue remodeling or alleviate proptosis or strabismus in the long-term (71). Recurrent disease is a common phenomenon after withdrawing corticosteroids (5). Therefore, corticosteroids are not the most suitable medicine for TED.

Mycophenolate is a drug that suppresses the proliferation of T cells and B cells (76,77). Mycophenolate was shown to be more effective and safer in a clinical trial than corticosteroids in patients with moderate-to-severe and active TED (77). In another clinical trial, researchers found that mycophenolate plus corticosteroids improved the rate of treatment response in moderate-to-severe and active TED cases (76). Although there are no data on subsequent rehabilitative surgeries and long-term follow-up (1), the risk-benefit ratio of low-dose mycophenolate alone or plus

intravenous corticosteroid injections indicates that it is safe and effective in TED (78). Hence, the 2021 EUGOGO guidelines recommend corticosteroids plus mycophenolate as the first-line management for moderate-to-severe and active TED (1). Radiotherapy is an alternative treatment in moderate-to-severe, and the response rate is ~60% (60). It is considered safe, but there are still some limitations, such as a remote carcinogenetic risk (1,59). In addition, some alternative medicines have been used in moderate-to-severe and active TED, such as cyclosporine and azathioprine (1,59,79).

1.3.4 Targeted therapy

The ideal medicines for TED should reduce the progression of inflammation, inhibit tissue remodeling, reverse fibrosis, and prevent the requirement for rehabilitative surgery. In addition, these medicines should have fewer side effects and an improved safety profile. To reach this goal, the mechanism of the disease needs to be elucidated. In recent years, several significant RCTs of targeted therapies in moderate-to-severe and active TED have been performed based on insight into the pathogenesis of TED (18-24). Rituximab is a drug that targets CD20 and causes B cell depletion and is the first targeted drug investigated in an RCT in patients with moderate-to-severe and active TED (23,24). A clinical trial in Italy showed that, compared with intravenous corticosteroid injections, rituximab is more effective (69% versus 100%) in moderate-to-severe and active TED, and there were no recurrent cases. Only a few patients required rehabilitative surgery after 76 weeks (24). However, a clinical trial in North America reported that, compared with placebo, rituximab provided no additional advantage in improving clinical features at 24 or 52 weeks (23). A subsequent analysis found that a long disease duration, older age, male, and high TSHR antibody level may affect the drug's effectiveness (80). In addition, CD20 was not expressed on the surface of plasma cells and did not affect plasma cell antibody production (81). Hence, rituximab may be more effective in the early inflammation phase and is suitable for patients with a short duration of TED (81,82).

Tocilizumab is a targeted drug developed to block the IL-6 receptor (83). In a clinical trial in Spain, tocilizumab was demonstrated to improve the CAS in patients with corticosteroid-resistant TED, and the researchers recommended its use in these patients (21). However, data on the efficacy and safety of tocilizumab are not available

yet (1). Teprotumumab, a drug that blocks the IGF-1R pathway, is currently the targeted agent with the most potential in moderate-to-severe and active TED (30,84,85). Teprotumumab has been confirmed to improve proptosis, the CAS, diplopia, and quality of life in moderate-to-severe and active TED (20,22). However, teprotumumab was shown to have severe teratogenic side effects on the fetus in an animal study (86). Moreover, a recent case report found that teprotumumab may cause inflammatory bowel disease, which is an autoimmune disease, but the mechanism is still unclear (87). Currently, teprotumumab is only available in North America, although it has been approved by the Food and Drug Administration of the United States (88). In addition, the time for response to teprotumumab in some patients is longer than in others (89), and the mechanism is not yet understood. Furthermore, its high price is a disadvantage for widespread use. In summary, there are still many limitations with current immunotherapies and targeted therapies, although these drugs have provided several benefits for patients with moderate-to-severe and active TED. Therefore, further investigation of the mechanism of TED may help develop novel therapies and optimize management.

1.4 Pathophysiology

1.4.1 Pathological changes

The exact pathogenesis of TED remains unclear, although most researchers have considered TED an orbital inflammatory disorder related to autoimmune thyroid disease (2,3,5,6,25,26,28,33). Autoimmune T cells, B cells, and OFs are activated by unbalanced immune tolerance caused by multiple factors (including antigenic stimulation, environmental changes, and genetics) (90-92). The orbit exhibits inflammation, edema, tissue expansion, extraocular muscle enlargement, adipogenesis, and angiogenesis (3,25,93), leading to a series of clinical manifestations (6,11,13). Irreversible tissue remodeling is observed in the orbit as the inflammation subsides (32).

Histological and serum examinations have revealed that glycosaminoglycans are overproduced and deposited in the orbital connective/adipose tissue and interstitial cells of extraocular muscles (94-97). Glycosaminoglycans are one of the significant

proteoglycan complex substances in the extracellular matrix and are vital for the morphology and function of the tissue (94). Glycosaminoglycans are involved in multiple physiological processes, such as regulating cell proliferation, differentiation, and inflammation (97). However, the most crucial characteristics of glycosaminoglycans in TED are their hydrophily and polyanionic charge, which enable them to absorb many times their weight in water (3,31,94). The overproduction of glycosaminoglycans, particularly hyaluronan, is one of the most apparent pathological mechanisms in TED, leading to edema and expansion of the orbital tissue volume (3,25,31,33). The subsequent signs and symptoms mainly from the contradiction of the development of orbital volume and the strict confines of the bony orbit (26,31,33,98).

Another significant pathological change is the enlargement of extraocular muscles (9,25,56,99,100). Orbital imaging examinations found that nearly 70% of adult patients with GD present with enlarged extraocular muscles (3). Interestingly, extraocular muscle enlargement predominates in some TED cases, whereas other patients present with significant expansion of orbital connective/adipose tissue (101,102). In contrast to younger patients with TED, older patients exhibit less fat tissue expansion and predominately show the enlargement of extraocular muscles (25,103). Additionally, those <40 years old mainly show changes in adipose tissue (25). Therefore, the heterogeneity in the signs and symptoms in TED may result from the heterogeneity in OFs (102) or the different pathological changes that patients experience.

1.4.2 Orbital fibroblasts (OFs)

OFs are considered the central cells in the pathogenesis of TED (2,3,25,26,30,31,33). OF-mediated interaction with immune cells via the production of different cytokines and chemokines is the primary mechanism for maintaining orbital inflammation in TED (2,5,31,104). OFs are heterogeneous cells with mesenchymal stem cell-like functions (105), and they undergo proliferation and differentiation (2,5,32,105). The heterogeneity in OFs may determine the signs and symptoms of TED (102), and most pathological processes and clinical features in TED involve OFs. In TED, OFs are mainly involved in orbital inflammation, orbital tissue expansion, adipogenesis, fibrosis, oxidative stress, and targeted receptor-costimulatory cell interactions (31).

According to the surface antigen CD90 (known as thymocyte antigen 1, Thy-1), OFs are divided into CD90+ OF and CD90− OF subtypes in TED (5,90,106,107). The two subtypes of OFs have different functions in the fibrosis of extraocular muscles, accumulation of fat, and local orbital inflammation in TED. CD90+ is the main subtype in the orbital fat/connective tissue, whereas ~30% to 40% are CD90− (31,102). CD90− OFs mainly differentiate into adipocytes and cause adipogenesis (32,102,106), and this mechanism is activated by cytokines (such as IL-1 and IL-6) (108) and prostaglandin D2 (98). CD90+ OFs have a low potential to differentiate into adipocytes, but their primary function is to differentiate into myofibroblasts in the presence of transforming growth factor (TGF)-β and then cause orbital tissue remodeling and fibrosis (31,32,106). Therefore, the heterogeneity of TED, which mainly affects fat/connective tissue or muscles, depends on OFs and their signals. The proliferation of fibroblasts is considered a crucial factor in tissue remodeling and fibrosis (109). Autoreactive T cells in TED stimulate the proliferation of OFs (104,110). A study confirmed that TED OFs have a stronger proliferative ability than healthy OFs (111). Both types of OFs (CD90+ and CD90−) overproduce glycosaminoglycans when activated by cytokines and antibodies, causing edema and increasing the volume of orbital tissue (31,90,112). Therefore, glycosaminoglycan overproduction by OFs, OF proliferation, and adipocyte differentiation from OFs are the main factors contributing to the expansion of orbital tissue.

OFs also produce cytokines and chemokines, such as IL-6, IL-8, IL-16, and monocyte chemoattractant protein-1 (MCP-1), depending on different molecular pathways to maintain orbital inflammation (31,113). The elevated cytokines subsequently stimulate OFs to produce intercellular adhesion molecules and glycosaminoglycans that are also involved in local inflammation (2,25,108). Moreover, OFs can act as antigen-presenting cells (CD40) to interact with and activate T cells via CD40-CD40L signaling pathways and participate in orbital inflammation (31,33,110). Regarding the targeted cells of the essential receptors in TED, TSHR and IGF-1R have been found to be overexpressed on the surface of OFs and are involved in the progression of TED (114-116). Finally, OFs participate in the oxidative stress that plays a vital role in TED (117-120). Oxygen radicals induce the proliferation of OFs and the expression of 72-kDa heat shock protein, leading to the production of reactive oxygen species and oxidative stress (117,118,121). In contrast to healthy controls, a series of substances involved

in oxidative stress, including superoxide dismutase, superoxide anions, malondialdehyde, hydrogen peroxide, and glutathione reductase, are significantly increased in the OFs of patients with TED (117,119,120). Studies have confirmed that the antioxidant drug selenium reduces the proliferation of OFs and the production of glycosaminoglycans and hyaluronan, and benefits patients with mild TED (67,122). In addition, several antioxidant drugs, including beta-carotene, N-acetylcysteine, vitamin C, and melatonin, have been confirmed to have benefits in TED via their effect on OFs (122,123).

1.4.3 TSHR and IGF-1R

TSHR is a glycoprotein hormone receptor that belongs to the G protein-coupled receptor family (91,124). It consists of an ectodomain, ligand-binding site in the extracellular region, endodomain located in the intracellular region, and transmembrane domain (91,124). TSHR is mainly expressed on the surface of thyroid epithelial cells (32). GD is considered an autoimmune thyroid disease caused by autoantibodies against TSHR and T cell infiltration (7,8,32,125). An apparent phenomenon in TED is that it usually occurs within 18 months of GD diagnosis (33,126), and studies have shown that 13% to 69% of GD cases develop into TED (26,52,126). The close relationship between TED and GD supports the concept that both diseases may share a common mechanism, and TSHR is considered the autoantigen that participates in this process (26,33,127). The direct evidence of TSHR involvement in TED is that TSHR messenger ribonucleic acid (mRNA) was found in orbital tissues from patients with TED by Feliciello et al. using polymerase chain reaction (128), although other researchers were unable to amplify TSHR mRNA in OFs and muscle cells in TED tissues (129). Subsequently, several researchers have confirmed that TSHR protein is increased in the orbital tissue of TED by immunohistochemistry (IHC) and immunofluorescence *in vivo* or *in vitro* (114,130-138). However, most studies included a small sample size, and the exact role of TSHR in TED is still unclear (26).

IGF-1R, a tyrosine kinase receptor, is a member of the insulin receptor family (114,139). IGF-1R has been confirmed to participate in the proliferation, apoptosis, metabolism, survival, motility, and migration of several cell types (33,114,140), thereby maintaining normal physiology (140). An early study reported that TSHR antibodies

were detected in immunoprecipitated tyrosine kinase receptors, such as IGF-1R (25,141). Later, Weightman et al. found that autoantibodies from TED or GD patients interacted with IGF-1 binding sites on OFs (142). Tsui et al. found that IGF-1R was expressed on the surface of OFs in TED by immunofluorescence (114), and the level of IGF-1R protein was reported to be higher in patients with TED than in healthy subjects (114,130,143). IGF-1 has been confirmed to increase the adipogenesis of orbital fat-derived stromal cells in TED (144). *In vitro* studies revealed that interrupting the IGF-1R pathway inhibited signaling from immunoglobulins in GD, indicating that IGF-1R is an autoantigen that regulates the infiltration of T cells and the production of glycosaminoglycans in TED (145,146).

1.4.4 Mononuclear cells

TED is considered an orbital inflammatory disease, and mononuclear cells play a significant role in local inflammation (2,3,5,25,26,28,31,33,147). The infiltration of mononuclear cells in local orbital tissue is both focal and diffuse between extraocular muscles and orbital fat/connective tissue (25). Using IHC, a series of studies demonstrated that T cells, some T cell subtypes, B cells, macrophages, a few mast cells, and a few plasma cells infiltrate the orbit (9,25,55,148-156). In an early study, Weetman et al. (156) found that the orbit is predominately infiltrated by T cells and to a lesser extent B cells. Subsequently, Kahaly et al. (155) found that macrophages are also involved in the infiltration of mononuclear cells. Later, some T cell subtypes were found to play a role in this process (9,151-154). However, the mechanism of T cells, B cells, and macrophages in TED varies with different studies (9,26,55,148-152). Some results are even contradictory (26,55,149,150,152). A systematic review identified some limitations in these studies, such as absent normal controls or a small sample size (26). Therefore, the exact mechanism of mononuclear cells in TED is still unclear, and the roles of T cells, B cells, and macrophages in TED require further investigation.

1.4.5 T cell subsets and related cytokines

The role of T cell-mediated immunity in TED development is widely accepted (27-29). Original T cells are from hematopoietic stem cells in the bone marrow, and mature T cells need to migrate to the thymus to develop (157). T cells differentiate into CD4+

helper T cells or CD8+ cytotoxic T cells after undergoing positive and negative selection in the thymus (28). CD4+ helper T cells participate in cellular, humoral, and innate immunity (28,158), whereas CD8+ cytotoxic T cells are mainly involved in the immune defense against intracellular pathogens and tumor immunity (159,160). In previous TED studies, many researchers confirmed that the infiltration of T cells in the orbit is predominated by CD4+ helper T cells and to a lesser extent CD8+ cytotoxic T cells (9,149,152-155). CD4+ helper T cells maintain developmental plasticity. They can further differentiate into T cells subsets, such as type 1 helper T cells (Th1), Th2, Th9, Th17, regulatory T cells (Tregs), and follicular helper T cells (161), based on the pathways and cytokines they encounter (161-163). T helper cell subsets are involved in several infections and inflammatory diseases by secreting unique cytokines (164).

In previous studies, Th1 cells predominated in the early stage of TED and participated in cellular immunity, and Th2 cells were mainly involved in the inactive phase and related to humor immunity (3,33,165). Interferon-γ (IFN-γ) is the signature cytokine expressed by Th1 cells, whereas IL-4 secretion is a marker of Th2 cells (3,27,29,162). Many researchers have focused on the role of Th1/Th2 subpopulations and their associated cytokines in the mechanism of TED, but some results are inconsistent (28). Th17 cells, a newly discovered CD4+ helper T cell subset different from Th1 and Th2 cells, mainly produce IL-17A, which acts *in vitro* and *in vivo* as a potent inflammatory cytokine (162,166). The overproduction of IL-17A has been associated with several inflammatory disorders, including psoriasis, psoriatic arthritis, rheumatoid arthritis, and ankylosing spondylitis (28,167). Furthermore, it has become an important therapeutic target for an increasing number of chronic inflammatory diseases (28,166-168). Recently, IL-17A has been found to be increased in the serum of patients with TED (29,169). IL-17A and its related cytokines (TGF-β, IL-6, IL-1β, and IL-23A), which promote pathogenic Th17 cell differentiation from naive T cells and maintain effector Th17 cells (162,170), have been reported to be increased in TED orbital tissue (171).

1.4.6 Chemokines and B cell-activating factor (BAFF)

Chemokines are a group of small proteins, associated with ~50 endogenous molecules and 20 related receptors (172,173). They play a crucial role in the maturation and migration of leukocytes (173,174) and participate in several inflammatory diseases (175). Regulated upon activation, normal T cell expressed and secreted (RANTES) is

a C-C chemokine produced by diverse cells, such as T cells, dendritic cells, macrophages, and microglia (176). RANTES participates in a series of signaling pathways, such as those that control chemotactic cell migration (177), and plays a crucial role in the inflammatory reaction by promoting immunocyte migration to the sites of inflammation (178). Several inflammatory diseases are related to RANTES, such as rheumatoid arthritis and inflammatory bowel disease (178). IL-16 is a T cell chemokine characterized by its ability to attract a variety of CD4+ helper T cells (179). IL-16 is produced by different cells and participate in several autoimmune inflammation diseases, such as multiple sclerosis, bowel inflammation disease, and rheumatoid arthritis (180). Gu et al. (181) found that RANTES and IL-16 are elevated in the serum of GD samples, and Fang et al. (182) subsequently reported that both chemokines were increased in the serum and orbital tissue of patients with TED. Therefore, IL-16 and RANTES may participate in local inflammation by attracting lymphocytes to migrate into the orbit in TED (32,33).

MCP-1 is a major chemokine that regulates the migration and infiltration of monocytes/macrophages (173). It is secreted by different cells, such as fibroblasts, epithelial cells, and monocytes (183). In an *in vitro* study in TED, MCP-1 was overproduced by activated OFs when incubated with IL-1β and palmitate (113,184). In addition, compared with healthy controls, MCP-1 mRNA was increased in TED orbital tissues (150). Therefore, MCP-1 may be involved in the local orbital inflammation in TED. Because rituximab (anti-CD20, B cells) achieves a significant benefit in TED, the crucial role of B cells in TED is widely accepted (24,81). B cells act as antigen-presenting cells and produce antibodies to participate in TED (47,81). BAFF, a member of the tumor necrosis factor (TNF) superfamily, plays a major role in promoting B cell survival, maturation, and immunoglobulin production (81,185,186). BAFF has been reported to be involved in many autoimmune diseases, such as GD, autoimmune thyroid disorders, and rheumatoid arthritis (185-187). In TED, studies have found that BAFF is increased in the serum of patients (82,188). Furthermore, BAFF protein has been found to be overexpressed in the orbital tissue of patients with TED compared with that in healthy controls (186). However, the study only used six orbital tissue TED samples and did not divide the patients into active and inactive stages (26,186). Therefore, it is necessary to investigate the exact role of BAFF in TED.

1.4.7 CD40-CD40L pathway

Multiple molecular signals determine the initiation of the acquired immune system. The primary sign is the binding of antigens to T and B cell receptors. Numerous secondary signals are involved in the interactions between co-stimulatory molecules on T cells and B cells and their related ligands (189). CD40, a crucial co-stimulatory molecule, is present on the surface of antigen-presenting cells, such as B cells (189-192), and its ligand CD40L is expressed on the surface of several cells, including activated T cells, basophils, mast cells, eosinophils, and platelets (193,194). CD40 is a member of the tumor necrosis factor (TNF) receptor superfamily, whereas CD40L belongs to the TNF superfamily (195). CD40-CD40L are involved in the induction and proliferation of antigen-specific CD4+ T lymphocytes, activation of antigen-presenting cells, upregulation of co-stimulatory factor expression, macrophage activation, and cytokine production (196). CD40-CD40L binding is a crucial event for immunocyte interactions in the immune system (197). Immunocytes are activated by the CD40-CD40L pathway and produce cytokines and chemokines to participate in inflammatory diseases (197). CD40-CD40L have been confirmed to be involved in multiple autoimmunity diseases, such as systemic lupus erythematosus, lupus nephritis, rheumatoid arthritis, and multiple sclerosis (189,191). Soluble CD40 and CD40L have been demonstrated to be increased in autoimmune thyroid diseases (198).

A study confirmed that CD40 is also expressed on OFs in TED, and the level of CD40 was increased in TED samples compared with that in controls (113). It is well known that OFs are considered critical cells in TED. Activated T cells and OFs play a central role in TED by producing a series of cytokines and chemokines to maintain orbital inflammation (31). A study related to the CD40-CD40L pathway confirmed that the inflammation process involves CD40 expression on B cells and OFs and CD40L expression on activated T cells (110). Uncovering the importance of the CD40-CD40L costimulatory pathway in the mechanism of TED may lead to the identification of a novel therapeutic target.

1.5 Immunohistochemistry (IHC)

IHC is a classic experimental method widely used in clinical and basic medical research (199,200). IHC can be used to explore the distribution of targeted proteins in

healthy and diseased cells and tissues (201). The principle of IHC is antibody binding to targeted antigens in tissue sections and cells, and the binding of the antibody-antigen complex is visualized by chromogenic or fluorescent detection (199-203). IHC can be generally classified into two types depending on tissue preparation: IHC-formalin-fixed, paraffin-embedded (FFPE) and IHC-frozen (200,204,205). Both FFPE and IHC-frozen have advantages and disadvantages. IHC-FFPE is widely used because of the advantages of not requiring expensive equipment and easy storage (201,206). FFPE tissue blocks can be cut at room temperature, the slides of tissue sections can be stored long-term at room temperature, and the morphology of FFPE tissue is better preserved than that of IHC-frozen tissues. However, the epitopes of FFPE tissues are masked during fixation, and some epitopes may be destroyed during antigen (epitope) retrieval (203). IHC-frozen preserves the antigen, but IHC-frozen tissue blocks need to be cut using a cryostat and usually stored at a lower temperature short-term (205). In addition, ice crystals can affect the morphology of the tissue. Therefore, investigators can decide the tissue preparation method based on the experimental condition and the features of target antigens.

Many commercial primary antibodies are available worldwide. A crucial decision is whether to select a monoclonal antibody or polyclonal antibody. Both types of primary antibodies have advantages and disadvantages. Monoclonal antibodies specifically bind to the antigen and reduce the background staining, decreasing signal detection (200,207). Polyclonal antibodies bind to multiple antigens, amplify signal detection, and are less affected by epitope changes but may cause cross-reactivity (200,207). Therefore, compared with polyclonal antibodies, monoclonal antibodies have higher specificity but lower sensitivity (200,207). In contrast, polyclonal antibodies have higher sensitivity but lower specificity than monoclonal antibodies (200,201,207). Another essential material in IHC is the secondary antibody. There are many labeling techniques for the secondary antibody in IHC, such as the avidin-biotin complex method, labeled SA-biotin method, and micro-polymer labeling method (200,202,208). In addition, immunofluorescence is a valuable immunochemical technique (209). In contrast to IHC, immunofluorescence has higher sensitivity and improved amplification in signal detection (209). However, immunofluorescence usually requires a specific microscope to visualize the immunostaining results. Nevertheless, the final goal of these techniques is to visualize the binding of the antibody-antigen complex and recognize the target proteins.

The assessment of immunostaining is the critical step in IHC. In previous TED studies, many investigators evaluated the immunostaining results manually. Specifically, they counted the positive cells in a particular (certain) field under microscopy (131,148-150,155,171,182,210). However, it is difficult to evaluate positive cells in some cases, such as when cytokines and chemokines are expressed throughout the entire tissue (149,171,182). In addition, tissue fibrosis is usually present in the orbital tissue of TED samples, and it is challenging to distinguish positive cells in these areas. Therefore, some investigators only show the molecules that are differentially expressed between patient and control tissues by presenting representative pictures of a few samples but without statistical analysis (171,182,210,211) or subjectively defining the positive staining of cytokines (149). Therefore, the manual assessment of immunostaining results is subjective and biased. To improve IHC techniques, many objective evaluation software programs have been developed. An accurate user-independent software program from Hungary has been extensively used in research (212,213).

2 Aim

TED is an orbital inflammatory disorder related to autoimmune thyroid disease. The management of TED is still unsatisfactory because of its complex and unclear pathogenesis. Uncovering the molecular mechanism underlying TED can provide further insight into the pathogenesis of the disease. Many *in vitro* studies have demonstrated that several pathogenic pathways, cytokines, and chemokines might be involved in the pathogenesis of TED. However, the actual environment *in vivo* is different from the situation *in vitro* in some cases. Therefore, using samples from patients with TED, such as serum, tears, and orbital tissue, is the most accurate way to reflect the clinical situation. Among these sample types, orbital tissue is the best sample to reflect the local inflammation, whereas tears and serum better present the circulatory environment. IHC analysis of human orbital tissue can reveal the local orbital immunity and correlate the microscopic changes with the macroscopic clinical manifestations (26,155). In the past three decades, many researchers have used the IHC method to investigate orbital tissues in TED and elucidate the pathogenesis of the disease (55,130,131,135,136,143,148-152,155,171,182,210,211,214). However, a recent systematic review identified several methodological limitations in these studies, including a small sample size, subjective assessment of the immunostaining results, and absence of active and inactive stages (26). As a result, the exact role of some molecules in the pathogenesis of TED remains unclear. Therefore, it is necessary to increase the sample size, use samples from well-documented patients, and objectively evaluate the immunostaining results with user-independent software programs in a blind and controlled manner. These methods are guaranteed to define the exact role of targeted molecules in the mechanism of TED. In addition, investigating novel molecules that have not been previously reported is important.

The study aimed to use 18 antibodies (molecules) against proteins involved in multiple pathways that contribute to the pathogenesis of TED (**Table 1**) to stain a large number of orbital tissue samples from patients with well-documented demographic and clinic data and investigate the exact role of these molecules in the pathogenesis of TED. Identifying the key molecules that play a crucial role in the mechanism underlying local inflammation in TED will help develop novel targeted treatments and optimize the management of TED.

Antibody	Function of the staining proteins
TSHR	Thyroid-stimulating hormone (TSH) receptor; key receptor in TED.
IGF-1R	Insulin-like growth factor 1 (IGF-1) receptor; key receptor in TED.
CD3	T cell marker.
CD20	B cell marker.
CD68	Macrophage marker.
IL17A	Pro-inflammatory cytokine; signature cytokine of innate immunity of Th17; induced orbital fibroblasts to differentiate into adipocytes and myofibroblasts; stimulated orbital fibroblast to secrete hyaluronan and glycosaminoglycans; exacerbated fibrosis.
IL-23A	Pro-inflammatory cytokine; maintained the production of IL-17A; stimulated orbital fibroblast to secrete hyaluronan and glycosaminoglycans.
IL-1β	Pro-inflammatory cytokine; maintained the production of IL-17A.
IL-6	Pro-inflammatory cytokines; stimulated naive T cells to differentiate into Th17 when cooperating with TGF-β.
TGF-β	Multifunctional cytokine; crucial role in T-cell regulation and differentiation; promoted fibrosis.
IFN-γ	Signature cytokine of Th1.
IL-4	Signature cytokine of Th2.
RANTES	Regulated upon activation, normal T cell expressed and secreted; chemokine; attracted T cells into the orbit.
MCP-1	Monocyte chemoattractant protein 1; chemokine; attracted monocytes/macrophages into the orbit.
IL-16	Chemokine; attracted T cells into the orbit, specifically targeting CD4 T cells.
BAFF	B cell activating factor, promoted B cell survival, maturation, and also the immunoglobulin production.
CD40	CD40 molecule marker; a costimulatory protein; on the surface of antigen present cells and B cell.
CD40L	CD40 ligand; binds to CD40; primarily on the surface of activated T cells.

Table 1. The function of the 18 stained proteins. The table presents the primary function of the 18 molecules in TED, not their overall functions.

3 Materials and methods

3.1 Participants and criteria

Forty-four patients with TED were enrolled from the Johannes Gutenberg University (JGU) Medical Center, Mainz, Germany, a prominent member of EUGOGO, which is the leading clinical and basic research group for Graves' Orbitopathy worldwide. The criteria for the clinical activity and severity in all patients with TED were based on the 2021 guidelines of the EUGOGO for the management of TED, which were approved by the European Society of Endocrinology (1). Clinical and serological evaluation of autoimmune-induced thyroid dysfunction followed the guidelines of the European Thyroid Association for the management of GD (4,7). Controls consisted of healthy individuals without any autoimmune orbital and/or thyroid disease, including 15 subjects from Shanghai Ninth People's Hospital of Shanghai Jiao Tong University School of Medicine, Shanghai, China, and one subject from the JGU Medical Center, Mainz, Germany.

This study followed the declaration of Helsinki, was approved by the local ethical committees and/or Institutional Review Board, and all patients provided their written consent.

3.2 Collection and preparation of orbital tissue

Forty-four consecutive orbital tissue samples were collected from patients with TED undergoing emergent or rehabilitative orbital decompression surgery in the Department of Ophthalmology, JGU Medical Center, Mainz, Germany. In comparison, sixteen control orbital tissue samples were collected in the Department of Ophthalmology at Shanghai Ninth People's Hospital of Shanghai Jiao Tong University School of Medicine (China) during blepharoplasty (n=15) or surgery for orbital trauma at the JGU Medical Center (n=1).

Forty-four TED and one control orbital tissue specimen from the Department of Ophthalmology, JGU Medical Center, were immediately placed in liquid nitrogen after surgical removal, frozen at minus 80°C, and stored in the Molecular Thyroid Research

Lab, JGU Medical Center, Mainz. The tissues were thawed at room temperature for 30 minutes before being fixed in formalin overnight and then embedded in paraffin at the Department of Pathology, JGU Medical Center. Fifteen controls tissues from China were prepared using the same method (FFPE) and made into paraffin tissue blocks, which were stored at room temperature.

Sixty paraffin tissue blocks were cut into two-micrometer-thick tissue sections using a microtome in the Department of Pharmaceutical Biology, JGU, Mainz, Germany. Paraffin tissue blocks were frozen for 1 hour on tissue block freezing machine before cutting to ensure that the tissue section remained flat after cutting. Tissue sections were placed on the surface of warm water (~45°C) in a water bath for 3–5 seconds to flatten any wrinkles. Next, the flattened tissue sections were picked up using microscope slides, and slides were dried at room temperature overnight and then stored in the slide box. Slides can be stored at room temperature long-term before immunostaining.

3.3 Equipment and materials

Equipment and material	Manufacturer/provider
Orbital tissue	Please see parts 2.1 and 2.2
Formalin	In-house, provided by the Department of Pathology, JGU Medical Center, Mainz, Germany
Paraffin	In-house, provided by the Department of Pathology, JGU Medical Center, Mainz, Germany
Paraffin tissue block	Prepared in the Department of Pathology, JGU Medical Center, Mainz, Germany and Shanghai Jiao Tong University School of Medicine, Shanghai, China.
Paraffin tissue block fixing plastic	In-house, provided by the Department of Pathology, JGU Medical Center, Mainz, Germany
Refrigerator	Bosch, Germany

Microtome machine	Leica, Biosytems Nussloch GmbH, modell RM 2125, designed in Germany, Made in China
Tissue block freezing machine	FRYKA, kältetechnik GmbH, Ohmstreet 4, D-73730, Esslingen, Germany
Electric heating water bath	Gesellschaft für, labortechnik mbH, Burgwedel, Germany
Microscopy slide	Cat. No. 631-0108, VWR international bvba, Leuven, Belgium
Slide box	In-house, provided by the Department of Pharmaceutical Biology, JGU, Mainz, Germany
Electronic balance	Precisa Gravimetrics AG, BJ 2200c, Switzerland
pH-meter	VWR, pHenomenal, pH 1100L, Ser. Nr. 21461690, Germany
Thermometer	VWR, Precision thermometer, Germany
Counting glass cup	VWR, 1612-3838, 500ml, Germany
Timer	Digital timer, Germany
Vortex mixer	Heidolph, REAX 2000, Germany
Electronic shaker mats	IKA-labortechnik, janke kunkel GmbH co.kg, Germany
Pipettes	Eppendorf, Germany
Pipette tips	Eppendorf, Germany
Glass jar	In-house, provided by the Department of Pharmaceutical Biology, JGU, Mainz, Germany
Heat-resistant plastic jar	In-house, provided by the Department of Pharmaceutical Biology, JGU, Mainz, Germany
Heating pressure cooker	Tefal Vitacuisine, model: SERE S06, made in China
Humid chamber	In-house, provided by the Department of Pharmaceutical Biology, JGU, Mainz, Germany

Cover glass	VWR, 100 pcs, 24 x 50 mm, cat. No. 631-0146, Germany
Mounting medium	Thermo Fisher Scientific, REF 1900333, 120ml, United Kingdom
Computer	Fujitsu, Germany
Panoramic DESK (scanner)	Serial PDESK 013405, 3DHistotech Ltd., Budapest, Hungary
Panoramic Viewer software	Version 1.15.2 DensitoQuant, 3DHistotech Ltd., Budapest, Hungary

Table 2. Equipment and materials. The table presents all equipment and materials used in the immunohistochemistry experiments.

3.4 Reagents

Reagent	Manufacturer/provider
Xylene	VWR, CHEMICALS, France
70%, 96% and 100% isopropanol	In-house, provided by the Department of Pharmaceutical Biology, JGU, Mainz, Germany
70%, 96% and 100% ethanol	In-house, provided by the Department of Pharmaceutical Biology, JGU, Mainz, Germany
Na2HPO4 (anhydrous)	CAS-No: 7558-79-4, Merck KGaA, 64271 Darmstadt, Germany
NaH2PO4 (anhydrous)	CAS: 7558-80-7, VWR, BDH, PROLABO, CHEMICALS, made in EC
NaCl (anhydrous)	CAS-No: 7647-14-5, VWR, BDH, CHEMICALS, Belgium
20X PBS-Tween 20	CAS: 9005-64-5, 0.2 M PBS, 1% Tween 20, pH 7.4, Germany
HCl solution	In-house, provided by the Department of Pharmaceutical Biology, JGU, Mainz, Germany

KOH (anhydrous)	Art.-Nr.: 26420, SCS GmbH-Am Burgweiher 3-53123 Bonn, Germany
KOH solution	10 M, distilled water 100ml with KOH 56.11g
Distilled water	In-house, provided by the Department of Pharmaceutical Biology, JGU, Mainz, Germany
Phosphate-buffered saline (PBS)-stock	Na2HPO4 21.8g, NaH2PO4 6.4g, NaCl 180g, Distilled water 1000ml, 20X PBS-Tween 20 10ml. Mix to dissolve and then adjust pH to 7.4 by KOH or HCl solution
PBS application solution	Twenty-fold dilution of PBS-stock in distilled water
Citric buffer	1% citrate buffer pH 6 in PBS, SIGMA, PCode: 1002722139, United States
Citric buffer application solution (antigen retrieval solution)	Hundred-fold dilution of PBS-stock in distilled water
Liquid blocker super pap pen	Daido Sangyo Co., Ltd. Tokyo, Japan
Primary antibody	Please see table 4
Antibody diluent	Thermo Fisher Scientific, 125ml, TA-125-ADQ, United Kingdom
Primary antibody dilution	Diluted the primary antibody with antibody diluent based on the optimal concentration of the antibody (please see table 4).
Mouse and rabbit specific HRP/DAB IHC detection Kit 1)Hydrogen peroxidase block 2)Protein block 3)Mouse specifying reagent (complement) 4)Goat anti-rabbit HRP-conjugate; 5)DAB substrate	Abcam, Micro-polymer, ab235466, United States

6)50X 3,3'-diaminobenzidine (DAB) Chromogen	
Fresh DAB reagent	30 µl DAB chromogen were added to 1.5 ml of DAB substrate
Hematoxylin-stock	VWR, Merck KGaA, Darmstadt, Germany
Hematoxylin application solution	Five-fold dilution of PBS-stock in distilled water

Table 3. Reagents. The table presents all reagents used in the immunohistochemistry experiment, including some solutions that need to be prepared before use and applied immediately.

3.5 Primary antibodies

Antibody	Type of antibody	Manufacturer	Catalogue number	Diluted	Source
CD3	Polyclonal	Thermo Fisher	PA5-32318	1:20	Rabbit
CD20	Polyclonal	Thermo Fisher	PA5-16701	1:20	Rabbit
CD40	Polyclonal	Thermo Fisher	PA5-32325	1:20	Rabbit
IL-17A	Polyclonal	Thermo Fisher	PA5-79470	1:50	Rabbit
IFN-γ	Polyclonal	Thermo Fisher	PA5-95560	1:50	Rabbit
MCP-1	Polyclonal	Thermo Fisher	PA5-80413	1:100	Rabbit
TGF-β	Monoclonal	Thermo Fisher	MA523795	1:30	Mouse
CD68	Monoclonal	Thermo Fisher	14-0689-82	1:50	Mouse
BAFF	Monoclonal	Thermo Fisher	14-9017-82	1:50	Mouse
RANTES	Monoclonal	Thermo Fisher	AHC1052	1:50	Mouse
TSHR	Monoclonal	Thermo Fisher	MA528136	1:100	mouse
IL-16	Monoclonal	Thermo Fisher	MA529348	1:200	Rabbit
IL-1β	Monoclonal	Protein tech	66737-1-Ig	1:50	Mouse
IGF-1R	Monoclonal	Protein tech	66502-1-Ig	1:100	Mouse
CD40L	Monoclonal	Protein tech	66238-1-Ig	1:100	Mouse
IL-4	Monoclonal	Protein tech	66142-1-Ig	1:400	Mouse
IL-23A	Monoclonal	Protein tech	66196-1-Ig	1:500	Mouse
IL-6	Monoclonal	Protein tech	66146-1-Ig	1:500	Mouse

Table 4. Primary antibodies. The information for 18 antibodies used in immunohistochemical staining and the diluted primary antibody concentrations (optimal concentration) used in immunostaining.

3.6 IHC staining procedure

Step	Procedure (all at room temperature)
1	Deparaffinizing in xylene and rehydrating in graded isopropanol, 2 x 5 min 100% xylene 2 x 1 min 100% isopropanol 2 x 1 min 96% isopropanol 2 x 1 min 70% isopropanol
2	Washing in PBS, 3 x 2 min
3	Antigen retrieval with warm 1% citrate buffer pH 6 in PBS at 100 ˚C for 20 min, cooled down for 20 min
4	Washing in PBS, 3 x 2 min
5	Blocking nonspecific binding by using endogenous peroxidase block reagent for 10 min
6	Washing in PBS, 3 x 2 min
7	Blocking nonspecific binding by using protein block reagent to block nonspecific binding for 10 min
8	Washing in PBS, 2 x 2 min
9	Incubating in primary antibody dilution for 1 hour at room temperature
10	Washing in PBS, 3 x 2 min
11	Incubating in mouse specifying reagent (complement) for 10 min. This step can be omitted when using primary rabbit antibodies
	Washing in PBS, 2 x 2 min
12	Incubating in Goat anti-rabbit HRP-conjugate reagent for 15 min
13	Washing in PBS, 4 x 2 min
14	Chromogen detection was used fresh DAB reagent for 10 min
15	Washing in PBS, 4 x 2 min
16	Washing in distilled water for 10 seconds
17	Counterstaining was performed with hematoxylin for 3 min
18	Washing in PBS, 3 x 2 min
19	Washing in running tap water for 10 min
20	Dehydrating in graded ethanol and cleaning in xylene, 2 x 1 min 70% ethanol 2 x 1 min 96% ethanol 2 x 1 min 100% ethanol 2 x 5 min 100% xylene
21	Covering staining slide with the cover glass and mounting medium

Table 5. IHC staining procedure. All 18 antibodies were used for immunostaining following this procedure. The optimal concentrations of 18 antibodies (Table 4) were used for all tissue section slides from 44 patients with TED and 16 controls. In addition, the primary antibodies were omitted for the negative control, and the antibody diluent was used instead.

3.7 Assessment

All the immunostaining slides were scanned by the Panoramic Desk (212,213) and evaluated by the user independent Panoramic Viewer software version 1.15.2 DensitoQuant (3DHistotech Ltd., Budapest, Hungary) in the Department of Pharmaceutical Biology, JGU, Mainz, Germany. This is an application that identifies the positive stain, based on an automatic color separation method through which individual positive pixels are counted and classified based on intensity and threshold ranges (brown color indicated positive staining, while blue counterstaining alone indicated negative staining). Quantification (percentage of positivity) was calculated by dividing the ratio of all positive cells to the total number of cells found in each of six independently annotated orbital tissue areas.

The evaluation method was selected for six interesting areas of orbital connective and adipose tissue, respectively in each slide. Subsequently, the same parameter of the DensitoQuant software was used to evaluate all slides, which were stained by the same antibody and following the software instructions to calculate the percentage of positivity. After defining the percentages of positivity of six areas from the orbital connective (OCT) and adipose tissue (OAT), the mean value of six areas was calculated as protein expression of the corresponding sample. The parameter should distinguish all the staining slides, where the optimal concentration of each antibody should differentiate all slides. The decision of the parameter was usually based on the staining experience and the literature. As long as the same parameter is used, all values are reproducible, independent of the operator, allowing for an automated, quick, and unbiased analysis of antibody expression. All immunohistochemically evaluations were performed in a blinded manner without any information pertaining to clinical stages, the activity of TED, and medical management. Once all immunostaining results were available, they were correlated with demographic, ophthalmic and clinical data.

3.8 Statistical analysis

The Statistical Package for the Social Sciences (SPSS) software program (version 26) was been used for statistical analysis. Two groups of TED patients and controls were analyzed by independent sample t tests if data were normally distributed and with non-

parametric tests (Mann Whitney U Test) in case of skewed distributions (215). For comparing three different groups of clinically active versus inactive TED and control, a one-way ANOVA test was used in case of equivariance while non-parametric tests (Kruskal-Wallis, 1-way ANOVA) were used, if skewed distributions were present (215). Significance levels of $P<0.05$ were considered as statistically significant. Graphs were made by Prism (version 9).

4 Results

4.1 Demographic and clinical data

Forty-four consecutive orbital tissues were obtained from patients with TED with various clinical activities and severity stages. Sixteen orbital tissues from 16 subjects without autoimmune, thyroidal, and endocrine diseases served as controls. Most patients with TED were female (37/44, 84%), with a median age of 56.5 years (25th/75th percentile, Q1/Q3 45/63 years). The median duration of thyroidal autoimmune disease in 44 patients with TED was 2 years (1.8/4.0 years).

According to the well-documented clinical data (**Table 6**), 44 patients with TED were divided into 22 active TED and 22 inactive cases based on the criteria of the 2021 guidelines of the EUGOGO. The median age of 22 active TED and 22 inactive TED was 55 (45/63) years and 57 (44.5/65) years, respectively, whereas the median CAS of 22 active and 22 inactive patients with TED was 4 (3/7) points and 1 (0/2) point, respectively. The median duration of orbital disease in 22 active and 22 inactive subjects was 1.5 (1.0/2.0) years and 4.0 (3/7) years, respectively. In addition, according to the 2021 guidelines of the EUGOGO, patients with TED can be divided into mild and severe disease groups. Among our samples, most active cases (95%) were classified as severe (95%, 21/22), and the majority of inactive cases were mild (77%, 17/22).

Prior to orbital surgery, all 44 patients with TED were treated with intravenous methylprednisolone pulses according to the EUGOGO guideline recommendations, and 13 of 22 subjects with inactive disease of a longer duration received orbital irradiation. The median time interval between steroid administration and orbital decompression surgery was two (Q1/Q3 1/5) and 17 (6/22) months in active and inactive TED, respectively. Of note, 10 of 22 patients with active TED had orbital surgery because of acute exacerbation of inflammatory eye signs and the development of optic neuropathy. At orbital surgery, all 60 subjects were biochemically euthyroid with serum-free thyroid hormone levels within the normal range. Ten of 16 controls were female with a median age of 32 (29/35) years.

4.2 Histopathology

Eighteen primary antibodies were used for the IHC staining of orbital tissue slices. In contrast to controls, the proliferation of connective tissue was significantly increased in TED orbital tissue samples, especially those from patients with a shorter disease duration and clinically active TED. Focal lymphocytic infiltration was observed in active specimens and to a much lesser extent in inactive cases and those with a longer disease duration but absent in control tissues. Angiogenesis was noted in all samples, most specifically in active tissues with a shorter disease duration. Tissue fibrosis was present in both active and inactive TED, mostly in patient samples with severe and active disease, but not in controls.

4.3 IHC analysis of orbital connective tissue

Compared with controls, 17 of 18 antibodies, including, TSHR ($P<0.001$), IGF-1R ($P=0.004$), CD3 (T cells, $P<0.001$), CD68 (macrophages, $P<0.001$), RANTES ($P<0.001$) and MCP-1 ($P=0.005$) (both chemokines that attract T cells and macrophages into the orbit), IL-23A ($P<0.001$) and IL-17A ($P<0.001$) (signature cytokines of the adaptive immune response/innate immunity), IL-6 ($P<0.001$), IL-1β ($P<0.001$), IFN-γ (Th1 cytokine, $P=0.002$), IL-4 (Th2 cytokine, $P<0.001$), CD40L (expressed on T cells, $P<0.001$), CD40 (expressed on antigen-presenting cells, OFs, and B cells, $P<0.001$), IL-16 ($P<0.001$), BAFF ($P<0.001$), and TGF-β ($P=0.002$), were highly expressed in the orbital connective tissue of patients with TED (**Figures 1–10 and Table 7**).

In comparing three different groups of clinically active versus inactive TED and controls, TSHR, IGF-1R, CD3, CD68 (**Figures 1 and 2**), and MCP-1 were overexpressed in clinically active TED ($P<0.001$) but not in inactive TED and controls. The expression of IL-23A and IL-17A (adaptive immune response), IL-6, IL-1β (**Figures 3 and 4),** RANTES (**Figures 5 and 6**), BAFF (**Figures 7 and 8**), and CD40L (**Figures 9 and 10**) was very high (patients versus control ratio >3, $P<0.001$), moderate (ratio >2), and low in active TED, inactive TED, and control tissues, respectively. CD40, IL-4, IL-16, and TGF-β (**Figures 7 and 8**) were overexpressed in both active ($P<0.001$) and inactive TED but not in controls. Mild-to-moderate B cell infiltration (CD20) was not different

between the active TED, inactive TED, and control groups (P>0.05). Compared with controls, IFN-γ was highly expressed in active TED (P<0.001) but not in inactive TED (P>0.05). There was no significant difference in IFN-γ between active TED and inactive TED (P>0.05). The magnitude of positive staining for the 18 antibodies tested in the orbital connective tissue of patients with TED (all stages), inactive TED, and active disease and controls is shown in **Table 7**. Smoking, age, gender, and thyroid function did not affect the IHC staining. In addition, the negative control of 18 antibodies, which contained the antibody diluent without the primary antibody, showed negative staining results (**Figure 11**).

4.4 IHC analysis of orbital adipose tissue

In contrast to connective tissue, orbital adipose tissue showed variable and rather moderate immunostaining (**Table 8**). Compared with controls, CD3 (P<0.001), IL-23A (P=0.003), IL-6 (P<0.001), RANTES (P=0.040), BAFF (P<0.001), IL-4 (P<0.001), CD40 (P=0.001), and CD40L (P=0.012) were highly expressed in the orbital adipose tissue of patients with TED. There were no significant differences in TSHR, IGF-1R, CD20, CD68, IL-1β, TGF-β, IL-16, MCP-1, and IFN-γ between TED and control samples (P>0.05).

In comparing three different groups of clinically active versus inactive TED and controls, TSHR, IGF-1R, IL-23A, and IL-1β (**Figures 1–4**) were overexpressed in clinically active TED (P≤0.035) but not in inactive TED and controls. CD3 expression was very high (P≤0.027), moderate, and low in active TED, inactive TED, and control tissues, respectively. IL-17A, IL-4, CD40, and CD40L were overexpressed in both active (P≤0.027) and inactive TED but not in controls. Compared with controls, RANTES was highly expressed in active TED (P=0.010) but not in inactive TED (P>0.05). There was no significant difference in RANTES between active TED and inactive TED (P>0.05). Compared with inactive TED, CD20 and MCP-1 were highly expressed in active TED (P≤0.049) but not in controls (P>0.05).

	TED (n=44)	CONTROL (n=16)	ACTIVE TED (n=22)	INACTIVE TED (n=22)
Age (year)	56.5 (45/63)	32 (29/35)	55 (45/63)	57 (44.5/65)
CAS (point)	N/A	N/A	4 (3/7)	1 (0/2)
Duration (year)	2 (1.8/4.0)	N/A	1.5 (1.0/2.0)	4.0 (3/7)
Interval (month)	N/A	N/A	2 (1/5)	17 (6/22)
Female ratio	84% (37/44)	63% (10/16)	91% (20/22)	77% (17/22)
Severe ratio	59% (26/44)	N/A	95% (21/22)	23% (5/22)
Steroid ratio	100% (44/44)	N/A	100% (22/22)	100% (22/22)
Euthyroid ratio	100% (44/44)	100% (16/16)	100% (22/22)	100% (22/22)

Table 6. Demographic and clinical data. Data are presented as the median (25th/75th percentile, Q1/Q3). CAS, clinical activity score; N/A, not available; Duration, duration of orbital disease; Interval, the time interval between steroid administration and orbital decompression surgery; Steroid ratio, the ratio of intravenous methylprednisolone pulses.

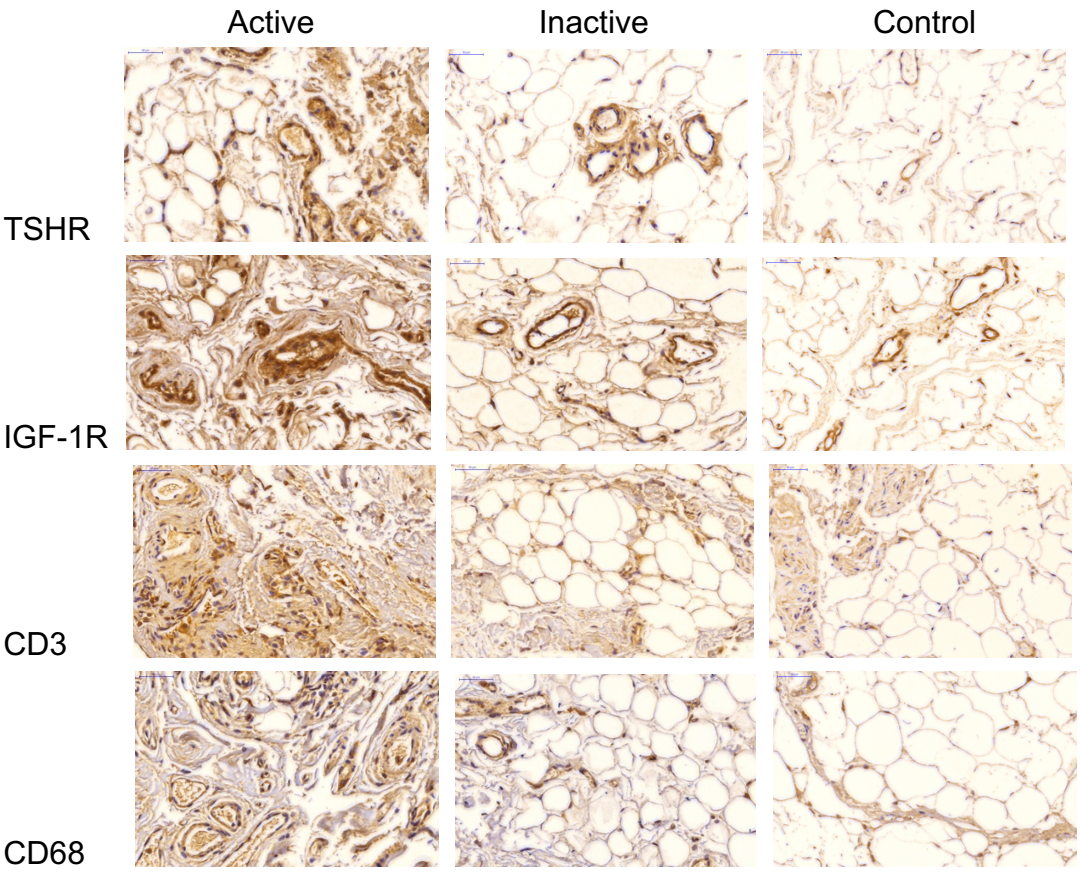

Figure 1. Immunohistochemistry staining results by TSHR, IGF-1R, CD3, and CD68 in the orbital tissue of patients with clinically active or inactive TED and controls. In contrast to inactive TED and controls, TSHR, IGF-1R, CD3 (T cells), and CD68 (macrophages) were overexpressed in the active stage of TED. Bars represent 50 μm.

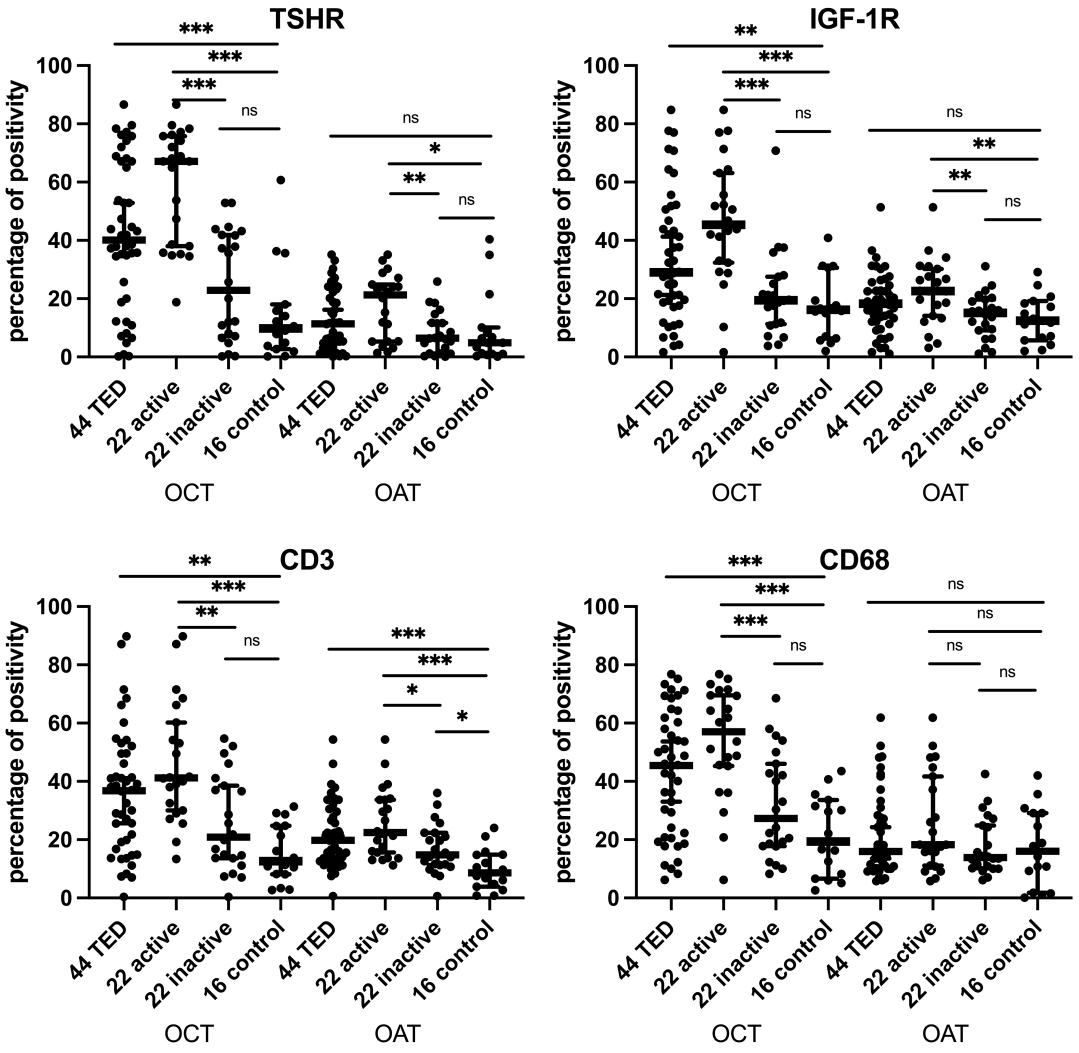

Figure 2. Percent positivity of TSHR, IGF-1R, CD3, and CD68 staining in different types of orbital tissues from patients with clinically active TED and inactive TED and control tissues. OCT=orbital connective tissue. OAT=orbital adipose tissue. In contrast to controls, TSHR, IGF-1R, CD3 (T cells), and CD68 (macrophages) exhibited a higher percent positivity in TED OCT. The percent positivity of TSHR, IGF-1R, CD3, and CD68 was increased in the orbital connective tissue of active TED versus inactive TED and controls. However, in contrast to connective tissue, adipose tissue presented moderate immunostaining. Only the percent positivity of CD3 was increased in the OAT of TED versus controls. Compared with inactive TED and controls, TSHR and IGF-1R were overexpressed in the OAT of active TED. CD3 expression was very high, moderate, and low in active TED, inactive TED, and control tissues. *P<0.05, **P<0.01, ***P<0.001; ns, not significant.

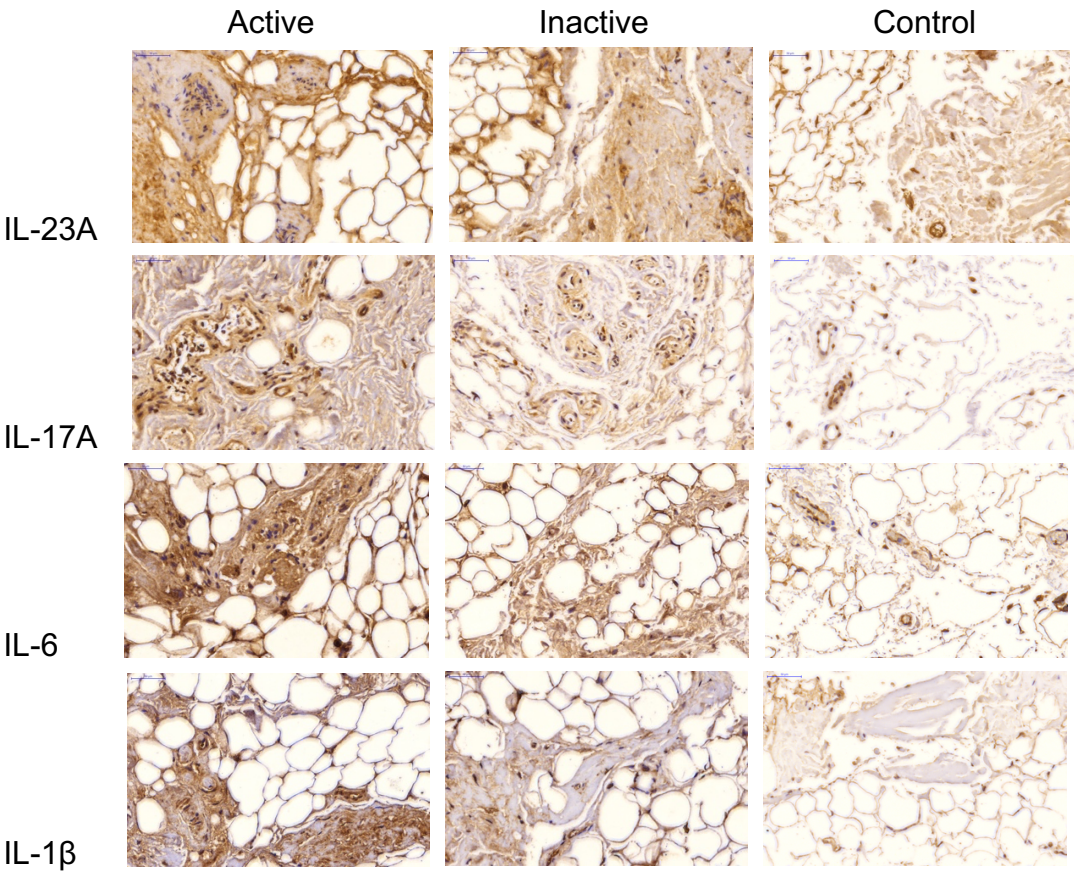

Figure 3. Immunohistochemistry staining by IL-23A, IL17A, IL-6, and IL-1β in the orbital connective tissue of patients with clinically active and inactive TED or controls. The pro-inflammatory signature cytokines of the adaptive immune response, most specifically IL-23A and IL17A, as well as IL-6 and IL-1β showed very high, moderate, and low staining in the orbital tissue of active disease, inactive TED, and control specimens, respectively. Bars represent 50 μm.

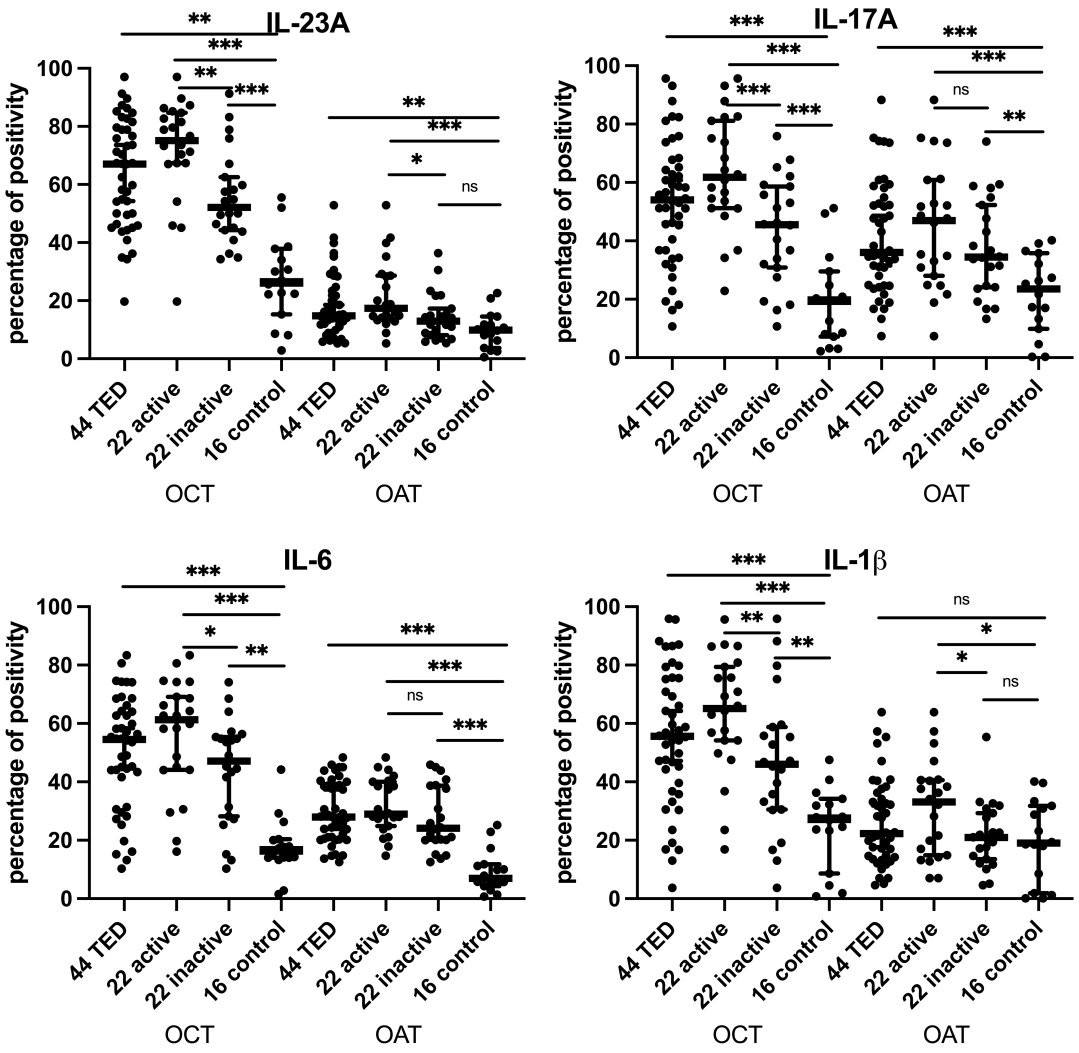

Figure 4. Percent positivity of IL-23A, IL17A, IL-6, and IL-1β in orbital tissues of patients with TED and controls. In contrast to controls, IL-23A, IL17A, IL-6, and IL-1β exhibited a higher percent positivity in TED OCT. IL-23A, IL17A, IL-6, and IL-1β showed a very high, moderate, and low percent positivity in the connective tissue of active TED, inactive TED, and control samples, respectively. For OAT, the percent positivity of IL-23A, IL17A, and IL-6 was increased in TED samples versus controls. In contrast to controls, IL-17A and IL-6 were overexpressed in the adipose tissue of both active TED and inactive TED patients. The percent positivity of IL-23A and IL-1β was increased in the OAT of active TED versus inactive TED and controls. *P<0.05, **P<0.01, ***P<0.001; ns, not significant.

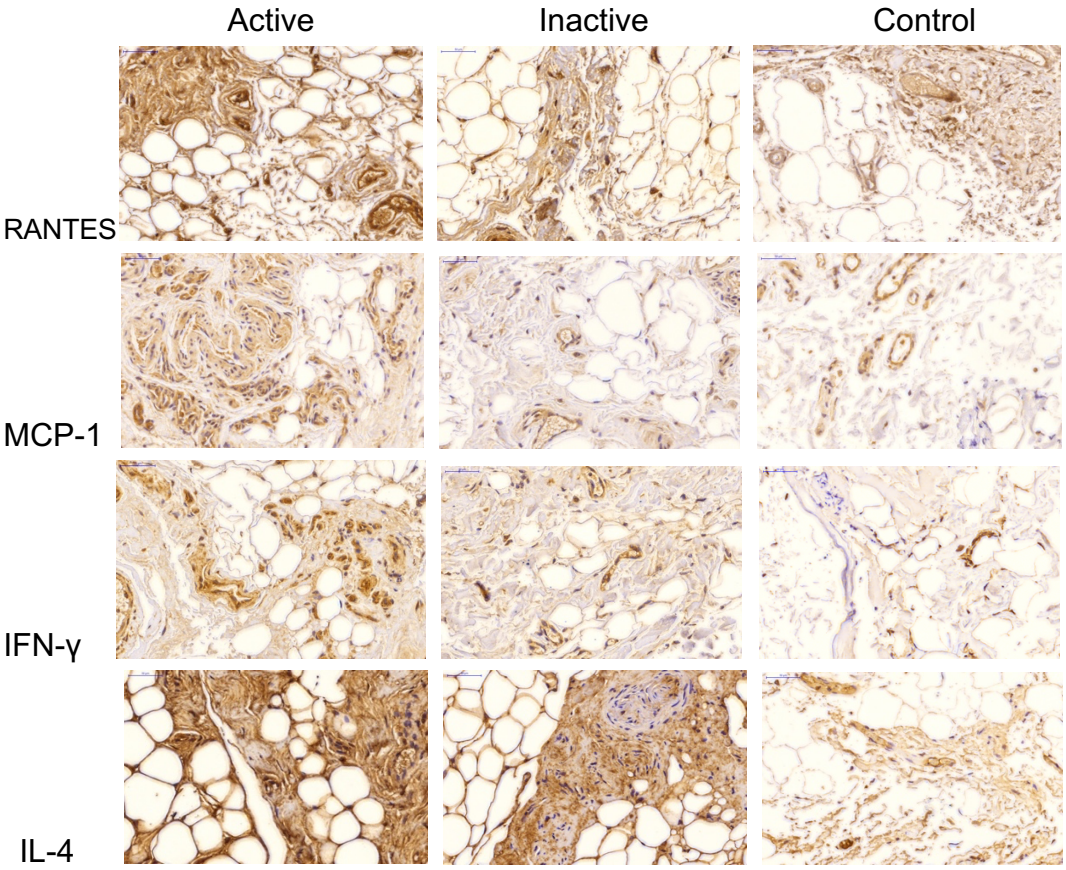

Figure 5. Immunohistochemistry staining by RANTES, MCP-1, IFN-γ, and IL-4 in orbital connective tissues of patients with clinically active or inactive TED and controls. The chemokine RANTES, which attracts T cells in the orbit, showed very high, moderate, and low staining in the active TED, inactive TED, and control specimens, respectively. The chemokine MCP-1, which attracts macrophages, was increased in the early stage of TED but not in inactive TED and controls. The cell-mediated immunity cytokines IFN-γ (Th1 cells) and IL-4 (Th2 cells) were overexpressed in active TED but not in control tissues. Bars represent 50 μm.

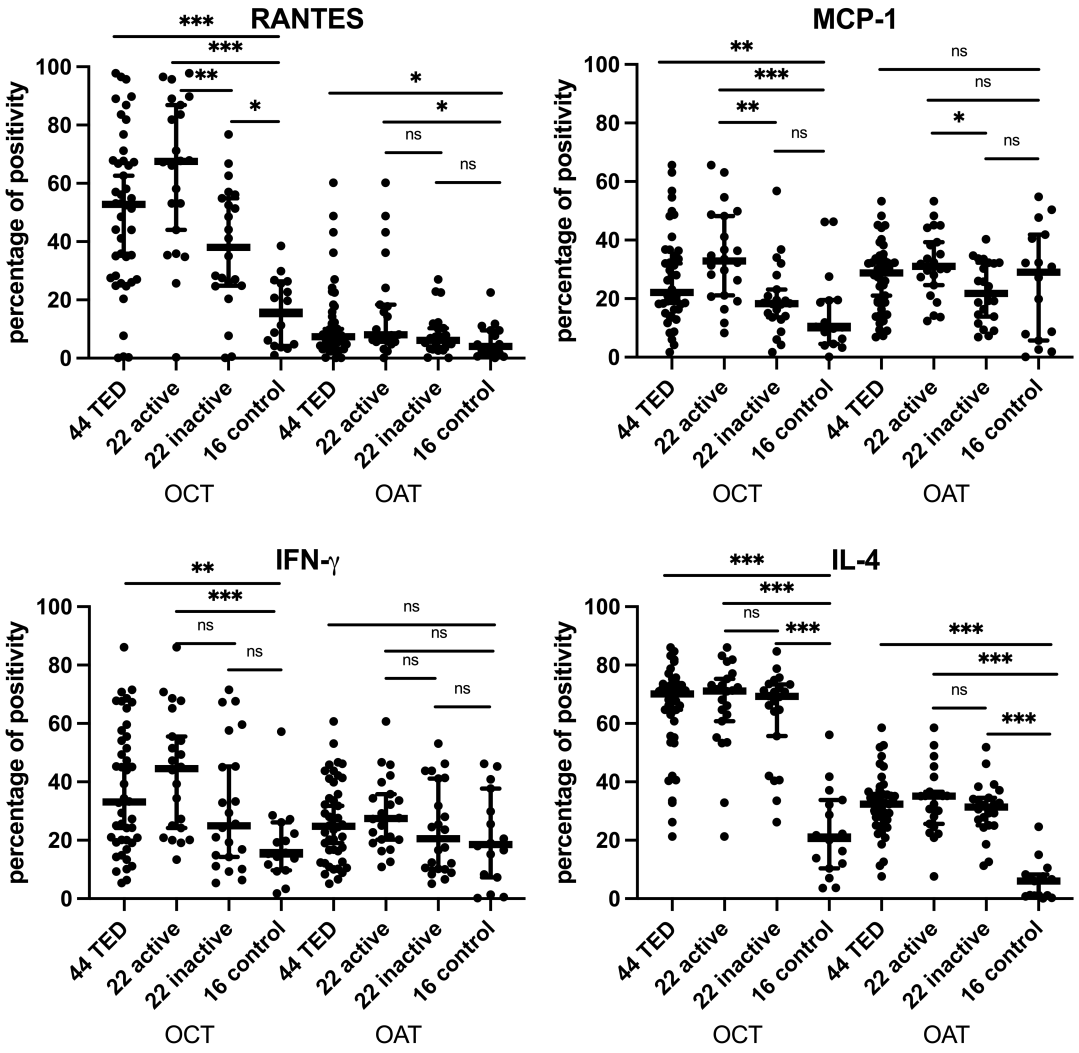

Figure 6. Percent positivity of RANTES, MCP-1, IFN-γ, and IL-4 in orbital tissues of patients with TED and control subjects. In contrast to controls, RANTES, MCP-1, IFN-γ, and IL-4 displayed a higher percent positivity in the OCT of patients with TED. The T cell chemokine RANTES exhibited a very high, moderate, and low percent positivity in the OCT of active disease, inactive TED, and control samples, respectively. The percent positivity of the macrophage chemokine MCP-1 in OCT was increased in active TED but reduced in inactive TED and controls. IFN-γ expression was high, low, and absent in active TED, inactive TED, and control OCT samples, respectively. In OCT and OAT, IL-4 expression showed a higher percent positivity in both active and inactive TED versus controls. *P<0.05, **P<0.01, ***P<0.001; ns, not significant.

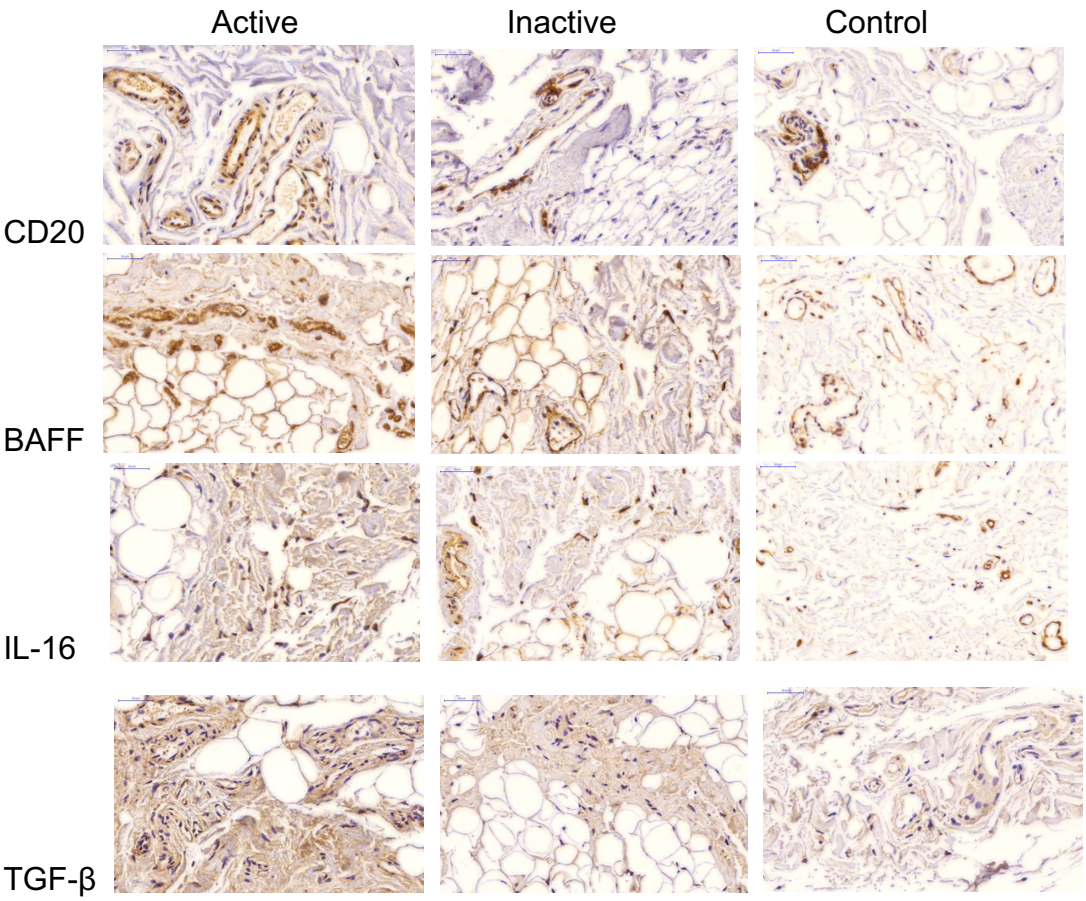

Figure 7. Immunohistochemistry staining results by CD20, BAFF, IL-16, and TGF-β in the orbital tissue of patients with clinically active or inactive TED and controls. Mild-to-moderate B cell infiltration (CD20) was not different between active TED, inactive TED, or control samples. BAFF exhibited very high, moderate, and low staining in the orbital connective tissue of active TED, inactive TED, and control specimens, respectively. IL-16 and TGF-β were overexpressed in both active and inactive TED but not in control tissue. Bars represent 50 μm.

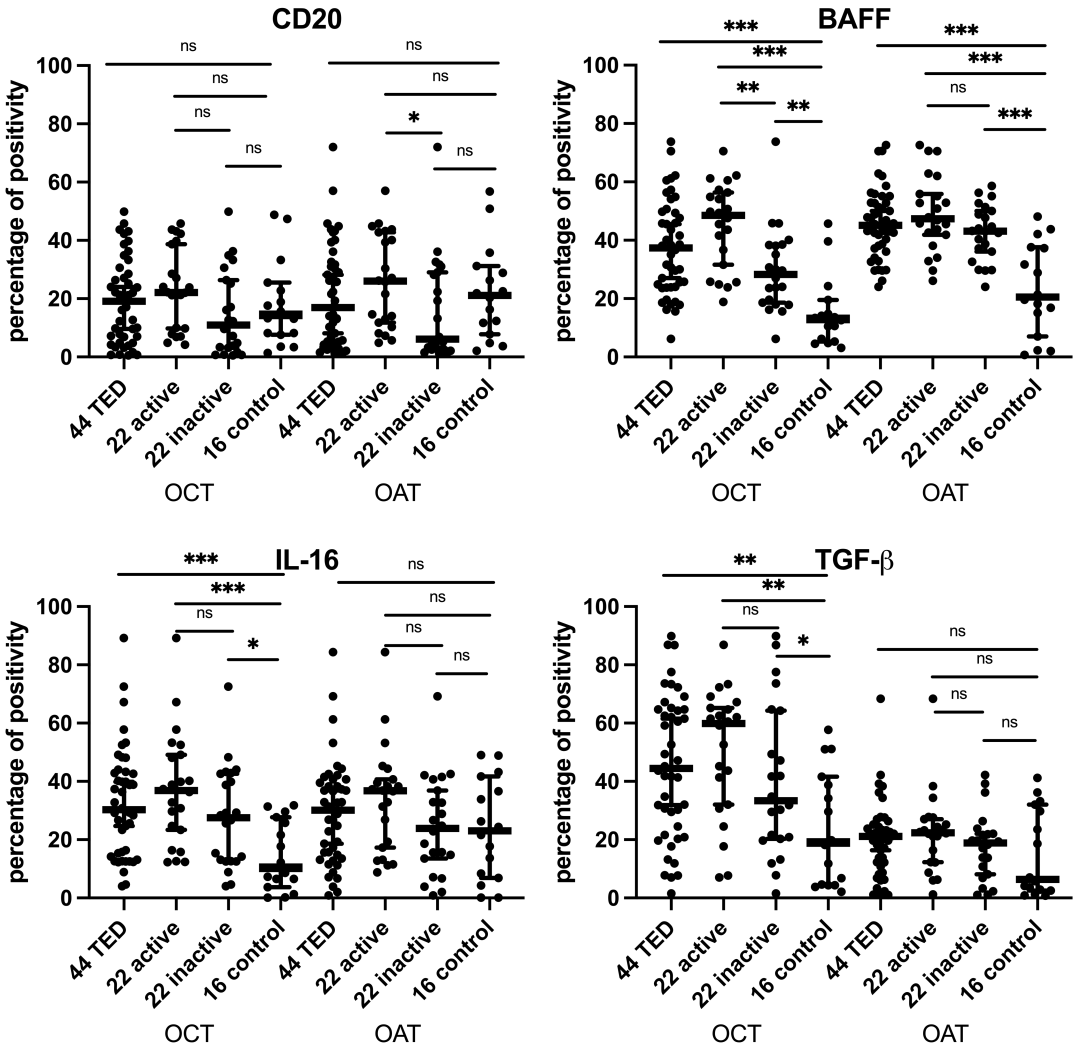

Figure 8. Percent positivity of CD20, BAFF, IL-16, and TGF-β in different orbital tissues from clinically active TED, inactive TED, and control patients. In contrast to controls, BAFF, IL-16, and TGF-β showed a higher percent positivity in TED orbital connective tissue. BAFF displayed a very high, moderate, and low percent positivity in the orbital connective tissue of active TED, inactive TED, and control samples, respectively. IL-16 and TGF-β exhibited a higher percent positivity in orbital connective tissues from both active and inactive patients with TED versus controls. *P<0.05, **P<0.01, ***P<0.001; ns, not significant.

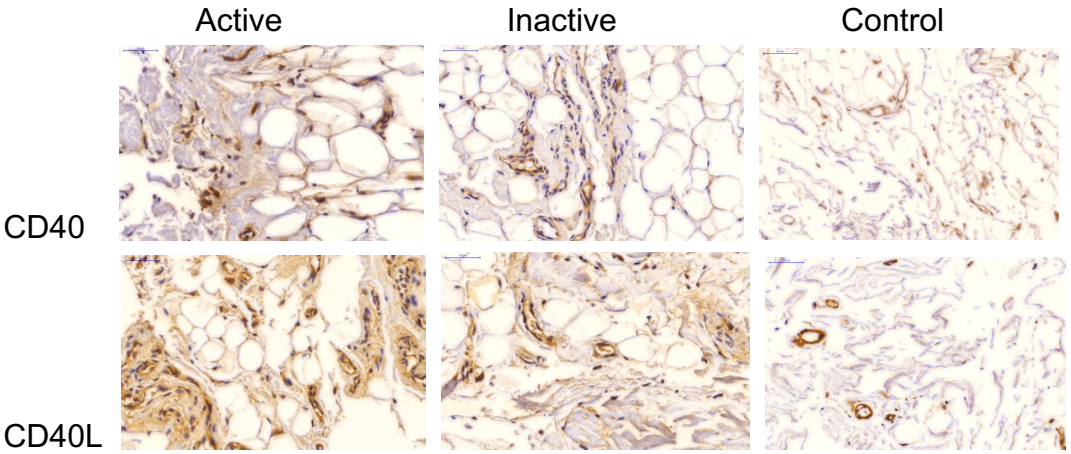

Figure 9. Immunohistochemistry staining by CD40 and CD40L in the orbital connective tissue from patients with clinically active or inactive TED and controls. CD40 was overexpressed in both active and inactive TED but not in control tissues. CD40L showed very high, moderate, and low staining in the orbital connective tissue of active TED, inactive TED, and control specimens, respectively. Bars represent 50 μm.

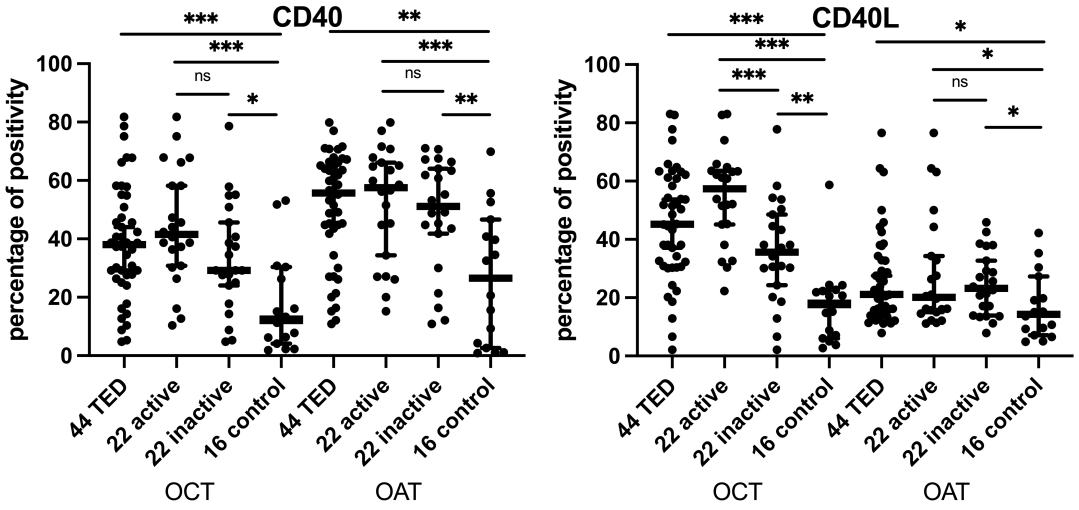

Figure 10. Percent positivity of CD40 and CD40L in the orbital tissues of patients with TED and controls. In contrast to controls, CD40 and CD40L showed a higher percent positivity in TED OCT. CD40 exhibited a higher percent positivity in OCT from patients with both active and inactive TED versus controls. CD40L displayed a very high, moderate, and low percent positivity in the OCT of active TED, inactive TED, and control samples, respectively. In OAT, CD40 and CD40L showed a higher percent positivity in patients with active and inactive TED than in controls. *P<0.05, **P<0.01, ***P<0.001; ns, not significant.

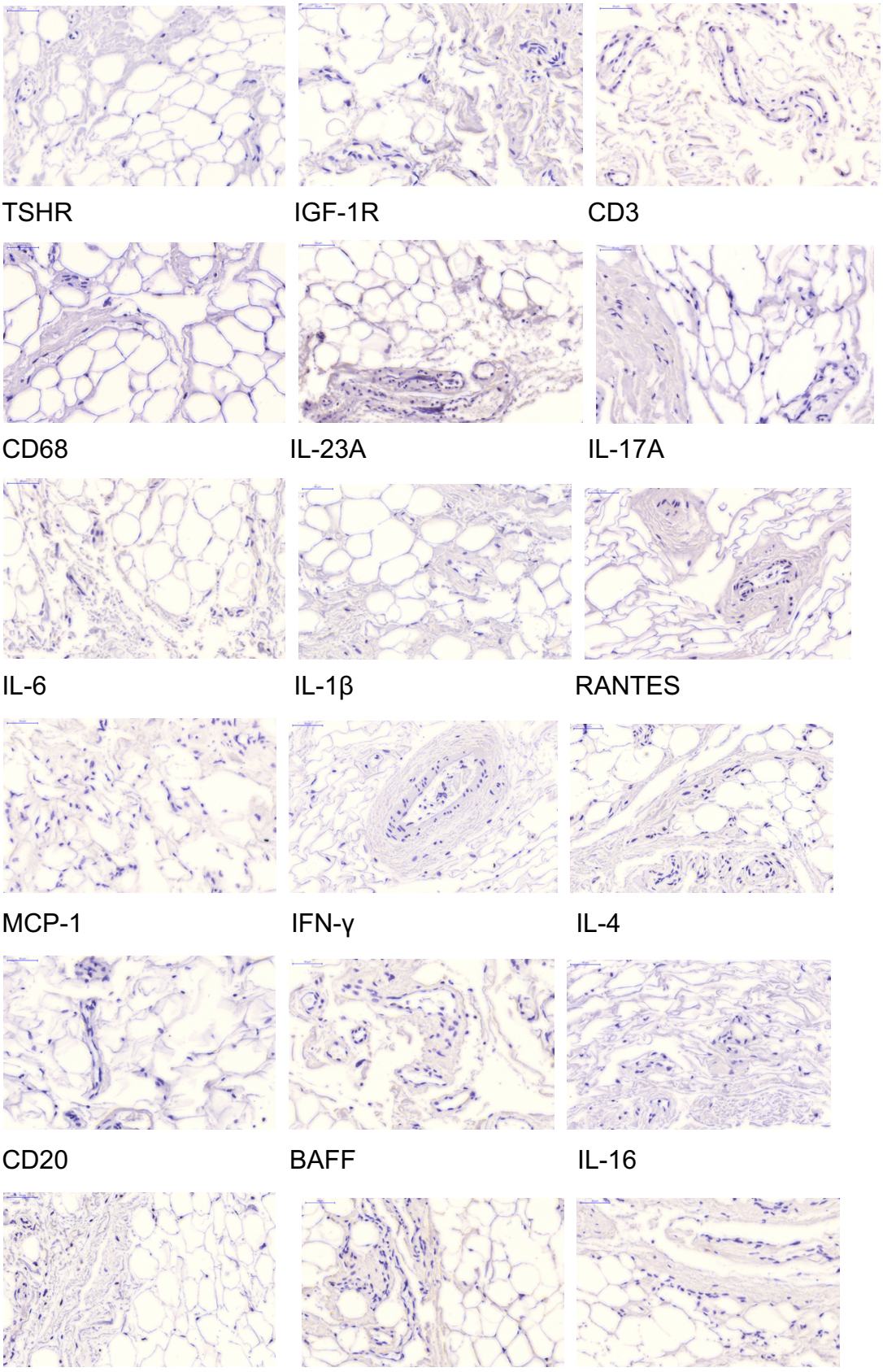

Figure 11. Negative control of 18 antibodies. The primary antibodies were omitted, and antibody diluent was used instead. All immunostaining results are negative.

5 Discussion

To the best of our knowledge, this is the largest IHC study of orbital tissue from patients with TED and healthy controls. This study used a larger number of specimens from patients with TED with well-documented clinical activity, severity, and disease duration. Moreover, the evaluation of immunostaining results was performed using a standardized user-independent software program in a blind manner. Furthermore, several antibodies that targeted candidate key players in TED were applied. In addition, after obtaining the assessment results, correlation analysis with ophthalmic and clinical data was performed. As a result, we observed several significant findings that have not been reported in previous IHC studies.

5.1 TSHR and IGF-1R

TSHR is considered a key autoantigen in the mechanism of TED and GD because of the close relationship between both diseases (26,33,127). This research confirmed that TSHR was overexpressed in TED versus controls, consistent with previous studies (130-138). Moreover, we observed that the immunoreactivity of TSHR was increased in the early and active stage of TED and rapidly reduced in the inactive phase and controls. Of note, in earlier studies, Boschi et al. (131) observed that TSHR-positive cells were overabundant in the early stage of TED and decreased in the inactive and chronic phase. However, they used extraocular muscles instead of orbital connective/adipose tissue and did not show any statistical analysis between the active and inactive groups. Recently, animal models of GD and TED have been successfully generated using mice immunized with the TSHR A subunit (216,217), further explaining the crucial role of TSHR in GD and TED. The noticeable morphological changes in model mice included increased collagen deposition and mucopolysaccharides (217) and clinical features of proptosis and conjunctival redness (216), consistent with the observations in the orbit of humans. In addition, many studies have reported that TSHR antibodies were related to the clinical activity in both TED and GD, and the TSHR antibody is a specific biomarker and major pathogenic autoantibody in both diseases (7,218-231). Thus, the results showing that TSHR was highly expressed in the early stage and expressed at lower levels in both inactive and control samples, combined with the critical role of TSHR and its ligands in animal

models and clinical correlations, indicate that TSHR may be the primary factor for TED initiation.

IGF-1R, another key autoantigen in TED, was reported after identifying IGF-1 binding sites on OFs in TED (142). In previous studies, IGF-1R protein was confirmed to be increased in the orbital tissue from patients with TED versus controls by immunofluorescence and IHC (114,130,143), and the expression of IGF-1R was correlated with the CAS (143). This is consistent with our research. However, these studies didn't differentiate between active and inactive TED. Our study was the first to observe very high IGF-1R immunoreactivity in patients with active TED and lower levels in both inactive TED cases and controls. Interestingly, the immunostaining results of IGF-1R are consistent with those for TSHR in this research. Many previous studies have confirmed that IGF-1R participates in TED, but the role of IGF-1R antibodies is still controversial (31). The level of autoantibodies against IGF-1R is similar in patients with TED and controls (14% and 11%) (232). The level of these antibodies does not correlate with the CAS of patients with TED, indicating that IGF-1R antibodies are not involved in the mechanism of TED (232). A recent study showed that the activation of the IGF-1R pathway is performed by the stimulation of TSH-R (immunoglobin/ auto-antibodies) against TSHR/IGF-1R crosstalk instead of direct interactions with IGF-1R (233). Currently, researchers believe that TSHR and IGF-1R form a complex, and this crosstalk plays a vital role in the mechanism of TED (233-237). The stimulation of TSHR activates two signal transduction pathways: one IGF-1R independent and one IGF-1R dependent (i.e., TSHR/IGF-1R crosstalk pathway with β-arrestin 1 acting as a scaffold (236)). Both pathways cooperate, resulting in the enhanced activation of OFs.

Given that IGF-1R is a crucial autoantigen involved in the pathogenesis of TED, blocking the IGF-1R pathway prevents the progression of the disease, and this has been demonstrated in several clinical trials (18-20,22). Recently, teprotumumab, an anti-IGF-1R monoclonal antibody, has been approved to treat active/severe TED by the Food and Drug Administration of the United States (88). TSHR peptides that block the TSHR pathway have been tested in GD and are considered a safe and potential novel effective therapy in GD (220). TSHR blocking monoclonal antibodies (K1-70) and small-molecule TSHR antagonists also hold great promise for the management of TED. The compassionate use of K1-70 has been recently reported in a metastatic

follicular thyroid cancer patient with co-existing GD and TED (238). K1-70 therapy resulted in the initial stabilization of malignant lesions, dramatic reduction in stimulatory TSHR-Ab activity, and significant improvement in TED. Therefore, similar to targeting IGF-1R, blocking TSHR in TED using monoclonal antibodies and small molecules may become the primary targeted therapy in TED in the future.

5.2 Mononuclear cells

The orbit is considered an environment prone to inflammation in autoimmune thyroid disease (93). In clinical practice, patients with TED usually respond well to immunotherapies in the early and active stages but poorly in chronic and inactive phases of the disease (55,56). This phenomenon may be related to the infiltration of mononuclear cells. However, the extent to which mononuclear cells play a significant role in TED is unclear (147). Our immunostaining results indicated that T cell (CD3) and macrophage (CD68) expression were higher in patients with TED than in controls. The result was consistent with previous studies (26,148-150,155). Furthermore, we found that T cell and macrophage infiltration in orbital connective tissue were increased in the active stage and absent in the inactive phase and controls. This is consistent with the findings from a recent Italian single-center study reporting a positive correlation between orbital T cell infiltration and CAS (55). Regarding macrophages, our result was different from that reported by Chen et al., who found that macrophage infiltration was not reduced in patients with TED with a long disease duration (150). We further analyzed the duration of TED in their manuscript and found that the disease duration in their samples (5.1 years) was longer than that for ours (2 years). It is well known that inactive TED usually occurs in patients with a disease duration of more than 2 or 3 years based on Rundle's Curve (10,44,54). Therefore, the long duration of TED might have affected the results of Chen et al. (150). The marked infiltration of T cells and macrophages and elevated chemokines (RANTES and MCP-1) in tissues from patients with active TED emphasize the relevant roles of cell-mediated immunity (153,154) and macrophages (155) as critical players in the early inflammatory phase of the disease and confirms the findings from the previous report. Thus, the observation that T cells and macrophages were the main infiltrating immunocytes in the inflammatory stage of TED but reduced in the inactive stage explains the phenomenon that patients treated with immunosuppressive therapy respond well in the active stages but poorly in the inactive phases.

In the present work, only a few samples of TED showed mild-moderate B cell infiltration (CD20), and there was no difference between TED and control tissue or between active and inactive TED. Our results are consistent with those reported by Eckstein et al. (152) and Pawlowski et al. (149). Eckstein et al. (152) analyzed a moderate sample size of patients with active and inactive TED and controls, and the duration of the disease in active and inactive TED was 1.3 years and 3.1 years, respectively. Similar to our study, all patients with TED in the research by Eckstein et al. (152) received steroid treatment. The mean time interval between steroid treatment and orbital decompression surgery was 5.2 and 28.4 months in patients with active and inactive TED, respectively (152). In the study by Pawlowski et al. (149), patients with TED were divided into mild (n=8) and severe (n=22) groups instead of active and inactive. However, based on the mean duration in mild (3.3 years) and severe (1 year) groups and the mean CAS in mild (2.2 points) and severe (8.5 points) cases (149), most instances of mild disease were from the inactive cases, and patients with severe disease were from the active group. In addition, all patients with TED received a steroid regimen. Nevertheless, Eckstein et al. (152), Pawlowski et al. (149), and our research did not find differences between patients with TED and controls or active and inactive TED cases. Our study observed that BAFF expression, the activation factor for B cells, was very high, moderate, and low in active TED, inactive TED, and control tissues, respectively. Therefore, the lack of difference in B cell infiltration but significant difference in BAFF among active TED/inactive TED/controls can be explained by the steroid administration.

5.3 T cell subsets and related cytokines

IFN-γ is the signature cytokine expressed by Th1 cells, whereas IL-4 secretion is a marker of Th2 cells (27,29). Compared with controls, IFN-γ and IL-4 were increased in TED. However, there was no significant difference between active and inactive TED, indicating that these two cytokines were sensitive but unspecific in TED, which mean that IFN-γ and IL-4 participate in the pathogenesis but do not play a significant role in TED. In this case, the present work could not explain whether the cytokines of Th1 or Th2 cells predominated in the early or later stages of TED. Combined with the clinical state of these samples, the lack of significant differences in the two cytokines between

active and inactive TED may be due to steroid administration, which affected the expression of some cytokines.

As anticipated in our previous review (26) and based on the current results, the adaptive immune response ("innate" immunity) with substantial overexpression of both IL-23A and IL-17A in TED orbital connective tissue appears to be extensively involved in the active phase of TED. Moreover, we observed that Th17-related cytokines (IL-6 and IL-1β) were elevated in TED samples versus controls, and the expression of IL-6 and IL-1β was very high, moderate, and low in the active TED, inactive TED, and control tissues, respectively. This is consistent with a previous study, although they used serum and tears (27). The significance of IL-17A, IL-23A, IL-6, and IL-1β immunoreactivity in the present work highlights the crucial role of innate immunity in the pathogenesis of the local orbital inflammation in TED. IL-17A, also known as IL-17, is a potent inflammatory cytokine (166). It not only participates in the defense against bacterial and fungal infections but is also involved in the pathogenesis of many chronic inflammatory diseases, such as multiple sclerosis, inflammatory bowel disease, psoriasis, and Graves' disease (166,167,239). Although many cells produce IL-17A, such as CD8+ T cells, natural killer cells, macrophages, and neutrophils, Th17 cells were the primary IL-17A-producing cells (166). Th17 cells have been found to be increased in orbital tissue and play a significant role in TED (27,28). IL-23A, mainly produced by dendritic cells and macrophages, plays a vital role in the mechanism of autoimmune diseases by maintaining the differentiation of Th17 cells and increasing the production of IL-17A (167).

IL-17A and IL-23A were found to be elevated in the serum of patients with TED by Kim et al. (169) and Shen et al. (29), and then Fang et al. (171) confirmed this finding in both serum and orbital tissue. In addition, Chen found that IL-17 expression was high, moderate, and low in the tears of active TED, inactive TED, and control subjects, respectively ($P<0.05$) (214). Additionally, Chen found that IL-17 was increased in the orbital tissue of patients with inactive TED versus controls ($P<0.01$) (214). Our result was consistent with the previous studies, although they used serum or tears (29,169,171,214). Here, we further demonstrated the different expression levels of IL-17A and IL-23A in local orbital tissue between patients with active and inactive TED. IL-17A stimulates the differentiation of OFs into pre-adipocytes and myofibroblasts, and IL-17A, IL-23A, and IL-6 stimulate OFs to secrete hydrophilic hyaluronan and

glycosaminoglycans (27,171). In addition, IL-17A promotes OFs to produce higher levels of RANTES, which may recruit lymphocytes from the systemic circulation into the orbit in patients with TED and maintain the local inflammatory reaction (182). Because IL-23/IL-17 signaling plays a significant role in TED, blocking this pathway may become a novel therapy in TED (27,28). Currently, two ongoing clinical trials are investigating the effects of blocking the IL-17A pathway in TED.

IL-6 is a pro-inflammatory cytokine produced by multiple cells and is usually increased in inflammatory conditions. In TED, IL-6 is overproduced by activated OFs (184). It induces B cell differentiation and autoantibody synthesis and promotes the expression of TSHR in OFs (47,168,240). Treg cells have been confirmed as anti-inflammatory cells by producing suppressive cytokines, thereby playing a significant role in autoimmune diseases (241). Naive T cells differentiate into Treg cells under the induction of TGF-β, whereas immature T cells differentiate into Th17 in the presence of IL-6 (162,163,166). Therefore, the differentiation of Th17 cells and Treg cells is related to the level of TGF-β and IL-6 (166). In previous studies, an unbalance in Treg and Th17 cells was suggested to be involved in the pathogenesis of TED (28,166,241). In the present work, we demonstrated that IL-6 participated in TED via the differential expression in the active stage, inactive stage, and controls. Studies have confirmed that blocking IL-6 alleviated the ongoing inflammation in TED (83). Because IL-6 regulates the balance between Th17 and Treg cells, interrupting the IL-6 signaling pathway by blocking the IL-6 receptor may increase Treg cells and restore balance in the immune system (166). Tocilizumab, a drug that blocks the IL-6 receptor, appears to provide benefit in patients with corticosteroid-resistant TED (21).

Previous studies showed that IL-1β combined with IL-23 induced γδT cells to produce IL-17 in the intestine, indicating that IL-1β participates in autoimmune inflammation (242,243). Our data showed that IL-1β was overexpressed in the active stage. Therefore, we presume that IL-1β may be involved in the mechanism of TED by maintaining the level of IL-17A. Of note, the hypothesis that the potential TED therapeutic agent Anakinra, which interrupts the IL-1β pathway by blocking IL-1β, may decrease the degree of inflammation, lymphocyte recruitment, and hyaluronic acid synthesis has been raised for many years (3). TGF-β contributes to fibrosis and tissue remodeling in TED (31,32,149). Our histology results revealed that tissue fibrosis was evident in both active and inactive TED samples but absent in controls. Our

immunostaining results also showed that TGF-β expression was increased in TED but decreased in controls, and the level of TGF-β was higher in both active and inactive TED. Therefore, this result may explain why fibrosis was extensive in both active and inactive samples but absent in controls.

5.4 Chemokines and BAFF

In our study, the expression of RANTES and IL-16 was higher in TED than in controls. This result is consistent with that reported by Fang et al. (182). Here, we demonstrated that RANTES expression was higher in the active stage of TED, decreased in inactive cases, and further reduced in controls. However, the expression of IL-16 was similar in both active and inactive TED. Therefore, our results confirmed that RANTES played a significant role in the progression of the inflammation in TED by attracting T cells to migrate into the orbit, thereby sustaining local inflammation during the active phase (8,181). Recent studies showed that the overexpression of MCP-1 in orbital inflammation activated the nuclear factor-kappa B complex pathway via several induced factors, including IL-17 and IL-1β (244), which were shown to be overexpressed in the orbital tissue of patients with TED in our study. In addition, MCP-1 has been confirmed to be overproduced by OFs in TED following treatment with CD40L (113). Our immunostaining research was the first to show that MCP-1 protein is highly expressed in TED samples versus controls. We also found that MCP-1 was upregulated in the active stage but expressed at low levels in inactive cases and controls. Interestingly, macrophages and MCP-1 expression showed similar trends in our data, indicating that MCP-1 may play a significant role in TED by promoting the migration of monocytes/macrophages into the orbit during the early stages of the disease. Consistent with Tang et al. (186), our results found that BAFF was overexpressed in TED samples versus controls. Moreover, we observed that BAFF was upregulated in orbital connective tissue during the active stage of TED and decreased in the inactive cases and controls. This result indicated that BAFF might participate in the primary mechanism of TED by promoting B cell survival and maturation. However, there was no significant difference in B cells between patients with TED and controls in several studies, which might be because of steroid administration. In addition, a recent study demonstrated that BAFF was increased in the serum of GD patients and proposed that blocking this pathway might be a novel treatment for GD (82,185). Indeed, BAFF plays a significant role in the pathogenesis

of TED; therefore, blocking this pathway may reduce the inflammation in TED (81,186). Belimumab, an anti-BAFF drug, is currently being tested in an RCT in TED (81).

5.5 CD40-CD40L pathway

In previous studies, the mRNA level of CD40 and CD40L has been reported to be increased in the orbital tissue of patients with TED (148), and CD40 protein was overexpressed in the orbital tissue of TED samples versus controls (210). Recent studies showed that CD40 and CD40L protein were also increased in the serum and tears of patients with TED (193,245). In the present work, CD40 and CD40L were highly expressed in orbital tissue in patients with TED versus controls, consistent with previous studies (148,193,210,245). CD40 was overexpressed in both active and inactive TED and absent in controls, whereas CD40L expression was high, moderate, and low in the orbital connective tissue of active TED, inactive TED, and control subjects, respectively. Although they used serum rather than orbital tissue, our results agree with those reported by Luo et al. (193).

It is well known that activated OFs play a significant role in the pathogenesis of TED via their proliferation, production of glycosaminoglycans, and differentiation into adipocytes and myofibroblasts, resulting in the expansion and remodeling of orbital tissue in TED (31,193). The CD40-CD40L pathway is involved in the interaction between OFs and autologous T lymphocytes in TED, and its signaling results in the activation of OFs and T cells (31,110,193). The activation of OFs by CD40 promotes the production of hyaluronan and prostaglandin endoperoxide H synthase-2, which heavier inflammation condition and increases the expansion of orbital tissue (246,247). In addition, the level of CD40 is increased in the presence of IFN-γ (113). In addition to interacting with CD40, CD40L binds to other non-CD40 receptors and activates a pro-inflammatory response (197). *In vitro* studies have shown that OFs produce chemokines, cytokines (such as MCP-1, IL-6, and RANTES), and intercellular adhesion molecules after being induced by CD40L, thereby promoting orbital inflammation and resulting in tissue remodeling in TED (192,240,248-250).

Given that CD40-CD40L signaling plays a significant role in the mechanism of TED, the hypothesis that blocking this pathway may be an effective therapy in TED has been raised for many years (3,115,192,247). Iscalimab, an anti-CD40 monoclonal antibody,

has been tested in Graves' hyperthyroidism in a clinical trial, and ~50% of patients responded (251). A study demonstrated that genetic polymorphisms in the *CD40* gene may contribute to the response to iscalimab in GD (252). Of note, the efficacy of this novel therapy was demonstrated in two GD patients with TED as it improved thyroid function and clinical eye features. However, this observation was from a study with a small sample size and uncontrolled study design (251). The benefit of iscalimab in TED requires further investigation. Several drugs targeting the anti-CD40L pathway, such as ruplizumab and toralizumab, have been tested in clinical trials but with failed results because of thromboembolic complications (190). A possible explanation for the thromboembolic complications is the expression of CD32a, which has been found on the surface of platelets in humans (190). The expression of CD40L was increased on activated platelets (253). The monoclonal antibody binds to CD40L and CD32a, resulting in platelet aggregation and thromboembolic complications (190). To address this, several new anti-CD40L targets have been developed and are being investigated in ongoing animal studies and clinical trials (254-256). We anticipate that anti-CD40L medications with an acceptable safety profile will be available in the future.

5.6 Limitations

Although our IHC study has identified several important molecules that play a major role in the pathogenesis of TED, there are still some potential limitations in this research. First, all orbital tissue samples from patients with TED were collected after steroid treatment, and there were no pre-treatment samples. Immunotherapy with steroids may modify the IHC pattern to a certain extent. Second, our study only recruited patients with TED with an indication of orbital decompression; therefore, mild TED or mild-to-moderate TED, which do not require orbital decompression, were not assessed. However, it is difficult to overcome the above two limitations as ethical approval is unlikely to be granted. Finally, we did not evaluate major histocompatibility complex expression in patient and control samples because this has been performed and reported numerous times (26,155).

6 Summary and conclusion

Currently, our study may be the largest IHC study of orbital tissue from patients with TED and healthy controls. As mentioned in the introduction, TED is an orbital inflammatory condition related to autoimmune thyroid disease, and its mechanism involves several pathogenic pathways and a network of infiltrating mononuclear cells, cytokines, and chemokines in the orbit. The management of TED is still suboptimal because of its poorly understood disease pathogenesis. Uncovering the key molecules that play a significant role in TED will help develop novel therapeutic strategies and optimize the management of TED. Here, we used 18 antibodies (molecules) against proteins involved in multiple pathways that contribute to the pathogenesis of TED to stain a large number of tissue samples from well-documented patients. Although some studies have confirmed that these molecules function in TED using mRNA samples, serum, or tears, the orbital tissue we used is the best sample to evaluate the local inflammation of the orbit. Although several IHC studies have found that some molecules were present in the orbital tissue of patients with TED, they did not differentiate between active and inactive TED, used a biased assessment method, included a small sample size, or did not perform statistical analysis. Therefore, the exact role of these molecules in TED remained unclear. However, we used a larger number of tissue samples from well-documented patients and differentiated between active and inactive TED. Therefore, our research is more significant, and we demonstrated several important findings.

Taken together, this multicenter, single-blind, controlled IHC evaluation of orbital connective and adipose tissue with a user-independent tool identified the functions of the following significant molecules in the pathogenesis of TED:

- TSHR and IGF-1R pathways played a vital role in the mechanism of TED. The stimulation of TSHR activates two signal transduction pathways, resulting in the enhanced activation of OFs. Blocking these two pathways is anticipated to be the major novel therapy in TED.

- T cells (CD3) and macrophages (CD68) were the main infiltrating immunocytes during the inflammatory stage of TED. The lack of difference in B cell infiltration

(CD20) but significant difference in BAFF among active TED/inactive TED/controls can be explained by steroid administration.

- The significance of IL-17A, IL-23A, IL-6, and IL-1β immunoreactivity highlighted the crucial of innate immunity in the pathogenesis of local orbital inflammation in TED. IL-17A stimulates the differentiation of OFs into pre-adipocytes and myofibroblasts, and IL-17A, IL-23A, and IL-6 stimulate OFs to secrete hydrophilic hyaluronan and glycosaminoglycans. Blocking these pathways may offer a novel therapy for TED.

- RANTES is involved in the inflammation in TED by attracting T cells to migrate into the orbital, whereas MCP-1 promotes the migration of monocytes/macrophages into the orbit. BAFF might participate in the primary mechanism of TED by promoting B cell survival and maturation. Targeting these molecules may achieve beneficial effects in TED.

- CD40-CD40L signaling participates in the mechanism of TED by mediating the interactions between OFs and T cells, and blocking this pathway for TED treatment is a promising option.

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8 Acknowledegment

9 Appendix

9.1 Publication

1, **Hai YP**, Lee ACH, Frommer L, Diana T, Kahaly GJ. Immunohistochemical analysis of human orbital tissue in Graves' orbitopathy. *J Endocrinol Invest*. 2020 Feb;43(2):123-137. DOI: 10.1007/s40618-019-01116-4.

2, **YP Hai**, MEM Saeed, KA Ponto, H Elflein, ACH Lee, S Fang, H Zhou, L Frommer, J Längericht, T Efferth and GJ Kahaly. Single blind, multicenter, controlled, immunohistochemically study of orbital tissue in autoimmune thyroid eye disease (in the submission of the journal of *Cellular & Molecular Immunology*).

9.2 Curriculum Vitae

Name: Yuan-Ping Hai

Date of birth: 01.01.1991

Place of birth: Sanya, Hainan, China

Education:

04.2018-now Johannes Gutenberg University Mainz,
Mainz, Germany, Pursuing a PhD degree.

09.2014-06.2017 South China University,
Hengyang, Hunan, China. Master of Medicine

09.2009-06.2014 Hunan University of Traditional Chinese Medicine,
Changsha, Hunan, China. Bachelor of Medicine.

Clinical practice:

02.2016-03.2018 Hainan Women and Children's Medical Center,
Residency, Paediatrics. Haikou, Hainan, China.

12.2014-01.2016 Hainan People's Hospital,
Residency, Internal Medicine. Haikou, Hainan, China.

06.2013-06.2014 Sanya People's Hospital,
Internships, Sanya, Hainan, China.

Practitioner's Certificate:

1. Physician's practice license of China for integrated Chinese and Western Medicine (2015)
2. Physician's practice license of China for Western Medicine (2017)

9.3 Supplemental tables

Two supplemental tables

	TED (n=44)	CONTROL (n=16)	*p1*	ACTIVE TED (n=22)	INACTIVE TED (n=22)	*p2*	*p3*	*p4*
TSHR	40.2 (19.1, 67.1)	9.8 (3.0, 17.5)	<0.001	67.1 (37.5, 75.9)	22.9 (7.0, 42.2)	<0.001	<0.001	0.107
IGF-1R	29.0 (17.9, 49.7)	16.2 (6.6, 27.8)	0.004	45.4 (31.6, 63.4)	19.5 (11.2, 27.6)	<0.001	<0.001	0.403
CD3	36.8 (17.4, 49.6)	12.6 (8.1, 24.8)	0.001	41.2 (29.8, 61.7)	20.9 (13.0, 39.2)	<0.001	0.002	0.082
CD20	19.2 (5.4, 30.1)	14.5 (7.8, 24.8)	0.880	22.1 (9.3, 39.0)	11.0 (2.8, 27.5)	0.258	0.058	0.530
CD68	45.5 (21.2, 61.5)	19.4 (7.0, 33.1)	<0.001	57.0 (43.1, 70.0)	27.3 (17.8, 47.0)	<0.001	<0.001	0.067
IL-17A	54.1 (36.8, 67.2)	19.5 (7.4, 28.3)	<0.001	61.8 (51.2, 81.4)	45.6 (30.1, 58.8)	<0.001	<0.001	<0.001
IL-23A	67.1 (47.2, 79.4)	26.3 (17.0, 36.9)	<0.001	75.2 (67.3, 84.8)	52.2 (44.2, 63.7)	<0.001	0.001	<0.001
IL-6	54.5 (34.0, 64.2)	16.6 (14.0, 20.3)	<0.001	61.3 (44.1, 70.4)	47.2 (28.0, 55.7)	<0.001	0.043	0.001
IL-1β	55.6 (37.5, 75.4)	27.4 (12.3, 33.7)	<0.001	65.2 (53.1, 79.8)	46.1 (30.5, 59.0)	<0.001	0.008	0.002
TGF-β	44.6 (22.3, 64.7)	19.0 (4.5, 40.9)	0.002	59.9 (31.7, 65.7)	33.4 (20.2, 64.4)	0.001	0.145	0.037
RANTES	52.8 (27.7, 67.9)	15.5 (4.4, 26.2)	<0.001	67.5 (42.0, 87.4)	38.1 (24.9, 55.2)	<0.001	0.005	0.011
IL-16	30.3 (14.5, 43.2)	10.4 (4.4, 27.2)	<0.001	36.9 (21.7, 50.0)	27.6 (12.6, 42.7)	<0.001	0.066	0.022
BAFF	37.4 (23.9, 49.7)	13.0 (5.5, 18.3)	<0.001	48.5 (30.2, 56.6)	28.3 (18.5, 38.4)	<0.001	0.001	0.002
MCP-1	22.1 (16.3, 36.0)	10.3 (5.0, 19.5)	0.005	32.9 (21.0, 48.3)	18.3 (13.4, 24.3)	<0.001	0.001	0.345
IFN-γ	33.1 (19.5, 55.2)	15.6 (10.2, 25.2)	0.002	44.6 (23.4, 57.9)	25.0 (13.5, 48.4)	<0.001	0.051	0.067
IL-4	70.1 (57.0, 73.5)	20.9 (10.8, 33.4)	<0.001	71.2 (59.4, 75.8)	69.3 (52.3, 73.4)	<0.001	0.499	<0.001
CD40	38.0 (26.7, 53.9)	12.3 (4.6, 29.4)	<0.001	41.5 (30.6, 60.2)	29.2 (22.5, 46.9)	<0.001	0.060	0.011
CD40L	45.2 (30.7, 61.1)	17.9 (6.3, 22.6)	<0.001	57.4 (43.4, 63.8)	35.7 (23.3, 49.0)	<0.001	<0.001	0.001

Table 7, Statistical analysis of orbital connective tissue by different groups. Data were presented as median (Q1, Q3). *p1*, the *p* value of 44 TED vs 16 control; *p2*, the *p* value of 22 active vs 16 control; *p3*, the *p* value of 22 active vs 22 inactive; *p4*, the *p* value of 22 inactive vs 16 control.

	TED (n=44)	CONTROL (n=16)	*p1*	ACTIVE TED (n=22)	INACTIVE TED (n=22)	*p2*	*p3*	*p4*
TSHR	11.4 (3.8, 23.1)	4.9 (1.0, 9.7)	0.082	21.3 (5.3, 25.5)	6.5 (1.2, 12.5)	0.018	0.005	0.795
IGF-1R	18.3 (11.5, 25.3)	12.5 (5.9, 19.0)	0.068	22.6 (13.9, 30.4)	15.1 (8.4, 19.9)	0.004	0.003	0.710
CD3	19.8 (13.0, 29.1)	8.6 (4.1, 14.9)	<0.001	22.5 (15.1, 33.8)	14.8 (11.2, 22.4)	<0.001	0.018	0.027
CD20	17.0 (5.2, 32.4)	21.1 (8.8, 30.6)	0.757	26.0 (11.4, 42.9)	6.1 (2.8, 29.5)	0.450	0.049	0.282
CD68	16.0 (10.8, 28.2)	16.0 (3.7, 29.1)	0.399	18.3 (11.0, 42.5)	13.9 (10.2, 25.5)	0.068	0.057	0.938
IL-17A	36.1 (24.8, 54.4)	23.5 (10.8, 34.9)	<0.001	47. 0 (27.2, 61.0)	34.5 (24.0, 53.0)	<0.001	0.164	0.008
L-23A	14.8 (11.2, 23.0)	9.9 (4.4, 13.8)	0.003	17.4 (13.8, 28.8)	13.0 (7.8, 18.4)	<0.001	0.025	0.112
IL-6	28.0 (20.6, 38.9)	7.0 (4.6, 11.7)	<0.001	29.0 (24.6, 40.1)	24.2 (20.1, 38.8)	<0.001	0.144	<0.001
IL-1β	22.3 (14.3, 35.8)	19.0 (5.2, 31.4)	0.216	33.2 (14.7, 40.7)	21.0 (13.2, 29.7)	0.019	0.035	0.667
TGF-β	21.1 (11.2, 25.0)	6.4 (2.6, 31.5)	0.219	22.4 (12.3, 27.2)	18.9 (7.8, 22.5)	0.110	0.250	0.579
RANTES	7.4 (4.1, 15.5)	4.1 (1.4, 9.5)	0.040	8.0 (5.6, 19.8)	6.1 (3.1, 10.7)	0.010	0.105	0.279
IL-16	30.2 (13.8, 40.4)	23.0 (7.3, 40.4)	0.371	36.8 (16.3, 41.6)	23.9 (11.9, 37.9)	0.084	0.090	0.860
BAFF	45.1 (37.4, 52.9)	20.5 (9.0, 37.5)	<0.001	47.4 (41.0, 57.4)	43.1 (35.2, 50.4)	<0.001	0.092	<0.001
MCP-1	28.8 (17.0, 33.7)	29.1 (6.3, 41.6)	0.678	31.1 (23.7, 40.5)	21.8 (13.3, 32.3)	0.155	0.025	0.505
IFN-γ	24.8 (13.5, 38.9)	18.5 (7.6, 35.8)	0.189	27.5 (19.9, 36.8)	20.6 (10.2, 41.2)	0.083	0.216	0.542
IL-4	32.4 (25.2, 36.4)	6.0 (1.0, 8.0)	<0.001	35.2 (25.4, 37.9)	31.4 (25.1, 35.2)	<0.001	0.299	<0.001
CD40	55.7 (36.3, 65.9)	26.6 (3.0, 45.2)	0.001	57.6 (32.6, 66.6)	51.2 (38.9, 64.6)	<0.001	0.536	0.002
CD40L	21.2 (14.6, 34.0)	14.3 (7.7, 25.4)	0.012	20.1 (14.9, 36.8)	23.2 (13.8, 34.0)	0.024	0.962	0.027

Table 8, Statistical analysis of orbital adipose tissue by different groups. Data were presented as median (Q1, Q3). *p1*, the *p* value of 44 TED vs 16 control; *p2*, the *p* value of 22 active vs 16 control; *p3*, the *p* value of 22 active vs 22 inactive; *p4*, the *p* value of 22 inactive vs 16 control.