Graves' disease: clinical manifestations, immune pathogenesis (cytokines and chemokines) and therapy.

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Abstract

Graves' disease (GD) is characterized by thyrotoxicosis, caused by the presence of circulating thyroid stimulating antibodies (TSAb), that are determinant also in the pathogenesis of its extrathyroidal manifestations [Graves' ophthalmopathy (GO), pretibial myxedema]. T helper (Th)1 immune response prevails in the immune-pathogenesis of GD and GO, during the active phase, when Th1 chemokines, and their (CXC)R3 receptor, play a key role. In GD, the existing treatments are not ideal for hyperthyroidism (long-term remission with anti-thyroid-drugs only in 50% of patients; while radioiodine and surgery cause hypothyroidism). In GD, antigen-specific therapy has been recently published, with the induction of T cell tolerance via an immunization by TSH-R peptides. In GO, rituximab and drugs targeting cytokines have been evaluated. Furthermore, teprotumumab (a human monoclonal anti-IGF-1R blocking antibody) showed to be very effective in GO patients.

Further researches are necessary to identify novel effective therapies targeting GD, or GO.

Keywords: Graves' disease; Graves' ophthalmopathy; CXCR3; CXCL10; Th1 immune response; teprotumumab.

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1. Introduction

Graves' disease (GD) is an organ-specific autoimmune disorder of the thyroid due to the presence of circulating anti-thyroid-stimulating-hormone receptor (TSH-R) stimulating autoantibodies that lead to hyperthyroidism. It is caused by the breakdown of immune tolerance against thyroid antigens, in particular against the TSH receptor (1,2), and it is characterized by thyrotoxicosis, the presence of serum antithyroid antibodies (ATA), as well as of autoreactive lymphocytes in the gland (3). The TSH-R, thyroid peroxidase (TPO), and thyroglobulin (Tg) have unusual properties ("immunogenicity") contributing to the breakdown of tolerance (2). The risk of GD is 3% for women and 0.5% for men, between 30 and 60 years of age, and it is the most common cause of hyperthyroidism in West Countries.

2. Clinical manifestations.

Clinical manifestations are associated with hyperthyroidism, but also with the autoimmune process. Thyroid hormones excess affect several different body systems, and for this reason, signs and symptoms associated with GD can vary strongly, and significantly influence the general well-being. Common symptoms are: tremor, heat sensitivity and warm, weight loss even if with normal eating habits, anxiety and irritability, enlargement of the thyroid gland (goiter), alterations in menstrual cycles, erectile dysfunction or decreased libido, fatigue, frequent bowel movements, palpitations, and others (4).

Fewer symptoms occur in older patients; in a study among >3000 consecutive patients with thyrotoxicosis it was reported that >50% of patients aged >61 years had fewer than three classic thyrotoxicosis symptoms (5), with atrial fibrillation as the most commonly associated feature. In 500,000 adults followed for >8 years, it was shown a 13% cumulative incidence of atrial fibrillation among people with thyrotoxicosis aged > 65 years (6).

Thyrotoxicosis rarely can cause thyrotoxic periodic paralysis (acute muscle paralysis and severe hypokalaemia), with a higher incidence in Asian men with thyrotoxicosis and is often a consequence of infection, alcohol, high carbohydrate load, or a hard physical activity (7).

Patients with thyrotoxicosis rarely manifest a life-threatening condition called thyroid storm, that is associated with deranged liver function, altered mental state, fever, agitation, features of cardiac failure, and tachycardia (8). Different events such as surgery, childbirth, infection, trauma or a poor compliance to the treatment, are able to precipitate the condition (9).

Graves' ophthalmopathy (GO) is present in about 30-50% of patients with GD.
Patients may have no ocular symptoms, may be distressed by the appearance of their eyes, or may be symptomatic. The common ocular symptoms that can occur individually or associated are: the sensation of a gritty or foreign object in the eyes; an excessive tearing (often made worse by exposure to wind, cold air, bright lights); diplopia; eye or retroocular pain or discomfort; blurring of vision; color vision desaturation; occasionally loss of vision. The characteristic signs of Graves' orbitopathy are proptosis (exophthalmos), tearing, and periorbital edema. The depth of the orbit, the degree of enlargement of the retroocular muscles and retroocular fibrous and fatty tissue, influence the degree of proptosis. The proptosis is usually asymmetric, but could be also symmetric, and a sensation of pressure behind the eyeballs can also occur. A periorbital edema commonly accompanies the proptosis, masking it. In more severe disease, there may be important conjunctival inflammation and ulceration from over exposure (10, 11).

Pretibial myxedema (PTM), also known as Graves' dermopathy or thyroid dermopathyis, is a rare infiltrative dermopathy complication of GD (12), whose incidence rate is of about 1-5%. This complication usually follows the ocular signs found in GD (13). It appears as a waxy, discolored induration of the skin (described as having a so-called *peau d'orange* appearance)

on the anterior aspect of the lower legs, that spreads to the dorsum of the feet; or as a non-localised, non-pitting edema of the skin in the same areas (14). In advanced cases, it can reach the upper trunk as well as upper extremities (face, neck, back, chest and ears).

Acropachy resembles clubbing of the fingers or toes and is present only in patients with dermopathy (15).

The association with another autoimmune disease is present in about 20% of GD patients. In a study, 3209 patients with GD (984 with GO), were prospectively evaluated for the prevalence of other autoimmune diseases vs. 1069 healthy control subjects, or 1069 patients with autoimmune thyroiditis (AT), or 1069 with multinodular goiter (MNG) (16) (with a similar iodine intake, and matched by gender and age). The results showed that 16.7% of GD patients had another associated autoimmune disease: Sjogren disease (0.8%); systemic lupus erythematosus and sarcoidosis (<0.1%); diabetes (type 1) (0.9%); celiac disease (1.1%); multiple sclerosis (0.3%); polymyalgia rheumatica (1.3%); rheumatoid arthritis (1.9%); chronic autoimmune gastritis (2.4%); vitiligo (2.6%). Three associated autoimmune disorders were present in 1.5% of GD. The prevalence of another autoimmmune disorder was higher (18.9%) in GO, than in GD without GO (15.6%). These results suggest that GD patients who develop new unspecific symptoms should be evaluated for other autoimmune disorders (16).

GD patients, in particular in the presence of thyroid nodules, have an increased risk of thyroid cancer. Tall Cell Variant of papillary thyroid cancer (a more aggressive form of cancer) was significantly more frequent in GD patients (17-20).

GD is a phasic disorder characterized during the active phase by hyperthyroidism, that can go in remission or after the medical therapy or in some cases also spontaneously. The recurrence of hyperthyroidism can be observed after weeks or decades of euthyroidism. The most frequent factors associated with recurrence are physical or psychological stressful events (15). Also, GO is characterized by the presence of moderate-severe inflammatory signs during the

active phase that can remit after therapy or spontaneously. The recurrence of active GO has been described to be frequently associated with the recurrence of hyperthyroidism in GD patients. The most frequent factors associated with the recurrence of GO are stressful life events (15), and smoke (21). Furthermore, large evidence has been accumulated on the detrimental effect of radioiodine therapy for hyperthyroidism of GD, on the appearance of GO ex novo, or the worsening of a previous present GO in patients with GD (15).

3. Pathophysiology

As already stated above, the onset of GD implicates a breakdown of immune tolerance towards the thyroid, through an autoimmune multifactorial process, involving environmental and endogenous factors in genetically predisposed subjects (22).

A large amount of evidence has shown that the active phase of GD, or GO, are associated with an immune prevalence of the Th1 immune response, while the inactive or later phases of GD and GO are associated with a switch from a Th1 to a Th2 immune prevalence (23,24).

Interestingly, considering the genes correlated with the risk of GD, about 70% with known effect, are implicated in T cell function, indicating the importance of T lymphocytes in autoimmune thyroid disease (AITD) pathogenesis (25,26).

In GD, autoimmune reaction causes the production of anti-TSH-R autoantibodies (TRAb) by B-cell clones, that infiltrate the gland. According to their actions on the TSH-R, TRAb antibodies can be classified as: thyroid stimulating antibodies (TSAb); thyroid blocking antibodies (TBAb); neutral antibodies (27,28). TRAb antibodies are implicated in GD pathogenesis and its extrathyroidal manifestations, i.e. GO and PTM/Graves' dermopathy. Hyperthyroidism is associated with TSAb. TSAb lead to similar downstream effects as the binding of TSH to TSH-R, such as the activation of adenylate cyclase and the following production of cyclic adenosine monophosphate (cAMP). This induces thyrocytes

proliferation, thyroid growth, and secretion of thyroid hormones (T4 and T3) (27). T4 (and especially T3) inhibits the secretion of TSH from the anterior pituitary via a negative feedback, and TSH is suppressed in GD hyperthyroidism. The role of TBAb and neutral antibodies is less understood in thyroid autoimmune pathophysiology (27). TBAb can bind to the A subunit of the TSH-R blocking the TSH action and its effects on the follicular cells, whilst the neutral antibodies bind to the receptor with no impact on cAMP generation or TSH binding (27-30).

4. Cytokines and chemokines in GD

4.1 Th1 chemokines

Chemokines (chemotactic cytokines) are small cytokines secreted by cells, that induce directed chemotaxis in nearby responsive cells. Some chemokines are considered homeostatic, whereas others are pro-inflammatory and attract immune effectors to sites of inflammation, during an immune response. Four principal chemokines subfamilies have been classified: CXC, CC, CX3C and XC. The chemokine receptor (CXCR)3 binds CXCL11, CXCL10, and CXCL9 (that are interferon-gamma dependent chemokine, Th1 chemokines) (31).

In the first phases of GD, CXCR3-expressing Th1 cells are recruited upon the production of CXCL10 by resident follicular epithelial cells, leading to the subsequent amplification of inflammation (32).

GD patients have higher serum CXCL10 levels compared to controls (matched by sex and age) (33). Circulating CXCL10 levels were similar in untreated patients in relapse of hyperthyroidism (earlier administered with MMI), and in untreated hyperthyroid GD patients of recent diagnosis, indicating that the active phases of GD are associated with elevated serum CXCL10, both in relapsing, or newly diagnosed, hyperthyroid patients (34).

Moreover, serum CXCL9 and CXCL11 levels were measured in 91 GD patients, 91 AT, 34 MNG, 31 toxic nodular goiter (TNG), and 91 healthy subjects (matched by age and sex) (35). CXCL9 and CXCL11 were higher in GD patients compared to the other subjects ($P < 0.05$). Hyperthyroid GD patients had higher chemokines levels than hypothyroid, or euthyroid ones, and GD patients with untreated hyperthyroidism than hyperthyroid, or euthyroid, GD ones administered with MMI. Newly diagnosed untreated hyperthyroid GD, and untreated patients with relapsed hyperthyroidism, had similar chemokines levels, suggesting that elevated circulating chemokines correlate with the active phases of GD (of new diagnosis or relapsing). Their decline in treated GD patients could be linked to the immunomodulatory action of MMI (35).

Furthermore, the effect of peroxisome proliferator-activated receptor (PPAR)-γ and of the IFN-γ and tumor necrosis factor (TNF)-α stimulated-Th1 chemokines secretion has been evaluated by other studies in primary GD thyrocytes *in vitro* (33,36). The release of these chemokines was absent at the basal level, and they were released in a dose-dependent manner by the treatment with IFN-γ, while no effects were observed by the treatment with TNF-α. The co-stimulation with IFN-γ+TNF-α induced synergistically the secretion of CXCL9, CXCL10, and CXCL11. The PPAR-γ agonists (pioglitazone or rosiglitazone) inhibited this effect dose-dependently, demonstrating that GD thyroid cells stimulated by cytokines take part into the reiteration of inflammation, by the secretion of Th1 chemokines, and PPAR-γ reduced this effect. The treatment with IFN-γ+TNF-α stimulated strongly the CXCL9 secretion, suggesting its leading role among the other chemokines (36).

In GD and control thyrocytes in primary culture, the presence of PPAR-α and PPAR-γ has been demonstrated and the effect of their activation has been evaluated after the treatment with IFN-γ and TNF-α (37,38). PPAR-α ligands inhibited the secretion of CXCL9, CXCL10, and CXCL11 stimulated by cytokines in a dose-dependent manner in both primary cell

cultures, with a higher inhibition on the CXCL9 secretion (90% with fenofibrate and 85% with ciprofibrate). The inhibition exercised by gemfibrozil on CXCL11 secretion was of 55%, while only of 40% on CXCL9. In thyrocytes, the inhibition of Th1 chemokines secretion was stronger with PPAR-α than PPAR-γ ligands, suggesting that PPAR-α can modulate the immune response (37,38).

Another study evaluated the relationship between functional polymorphisms in genes of certain chemokines and AITD pathogenesis (39). The following subjects were enrolled: 131 HT patients [54 with moderate-to-severe hypothyroidism with an age under 50 years and administered once-a-day with thyroxine (severe HT); 46 over 50 years of age, untreated euthyroid patients with HT (mild HT)]; 149 GD patients, 53 of whom in remission, and 59 with intractable GD; 99 healthy controls. The following polymorphisms were genotyped: IL8 -251T/A, Monocyte Chemoattractant Protein1 (MCP1)-2518G/A, IL16 -295T/C, Regulated upon Activation, Normal T cell Expressed and presumably Secreted (RANTES) -403G/A, -28C/G, IP10 -1596C/T and MIG rs2276886G/A. The MIG rs2276886 A allele and IL8 -251TT genotype were more common in AITD, while the RANTES -403AA and -28GG genotypes were less frequent. MIG rs2276886 AG genotype was uncommon in intractable GD patients, whereas HT patients showed more frequently the MCP1 -2518GG genotype. The age at onset in GD patients with -28CG and GG genotypes was higher vs. those with the RANTES -28CC genotype. These findings indicated the MIG rs2276886 AG genotype is associated with the GD intractability (39).

4.2 IL-21

A study investigated in PBMCs *in vitro* the expression of mRNA and protein of inflammatory cytokines, treated with recombinant human IL-21 (rhIL-21) (40). Treated GD and HT patients has higher levels of circulating IL-21 than those not treated, and IL-21+CD3+CD8 T cells

were significantly raised in PBMCs in HT vs. control subjects. The IL-21 mRNA in PBMCs was elevated in GD and HT, and IL-21 and IL-21 receptor (IL-21R) mRNA increased strongly in thyroidal tissues of HT patients. In presence of rhIL-21, cultured PBMCs from GD patients had stimulated IL-17A levels but decreased IL-4 production, whereas from HT patients, PBMCs had an increased production of IFN-γ. These results suggest that IL-21 play a pivotal role in the pathogenesis of AITD, since both IL-21 and IL-21R were found at high concentrations causing exaggerated immune response (40).

Another study investigated the role of IL-21 in the regulation of Th17/Treg cells in 28 GD patients of new diagnosis, 27 GD patients in remission (eGD), and 24 healthy subjects. PBMCs were isolated and cultured in presence/absence of recombinant human IL-21 (rhIL-21). In absence of rhIL-21, the expression levels of IL-22, retinoid-related orphan gamma t (RORγt), forkhead box protein P3 (Foxp3), IL-17, and IL-10 mRNA, and the IL-10 and IL-22 proteins were significantly higher ($P < 0.05$) in GD than in eGD and controls. On the contrary, after the rhIL-21 stimulation in GD, the RORγt, IL-17, and IL-22 mRNA levels and IL-22 protein levels increased, while Foxp3 and IL-10 mRNA levels and IL-10 protein levels decreased ($P < 0.05$). These data suggested that IL-21 can stimulate the differentiation of CD4+ T cells to Th17 cells and decrease Treg cell differentiation, contributing to the activation of the downstream immune response and GD pathogenesis (41).

4.3 IL-37

The role in GD of IL-37, belonging to the IL-1 family, that decreases local and systemic inflammation, is still unclear (42). The mRNA expression of IL-17, IL-6, IL-37, and TNF-α in PBMCs in 40 GD patients, and their circulating levels, have been evaluated, showing they were significantly higher with respect to controls. Serum IL-37 was tightly correlated with TSH, FT3, IL-17, FT4, IL-6, TNF-α, and TRAb. GD patients in the active phases of the

disease had IL-37 mRNA and serum protein levels significantly higher than those in the inactive phases, or healthy control subjects. In GD, IL-37 inhibited the production of TNF-α, IL-17 and IL-6 in PBMCs. These findings indicated that IL-37 exercises a protective role against inflammation in GD, repressing the production of proinflammatory cytokines, and it can be considered a novel target for the pathogenesis and treatment of GD (42).

4.4 IL-23

Two independent Chinese cohorts evaluated the genetic association between rs11171806 (IL-23A gene polymorphism) and susceptibility to GD (43): the Shanghai cohort (consisting of 712 GD patients and 705 controls), and the replication cohort from Xiamen Island (with 433 GD patients and 410 controls). GD patients had significantly higher circulating IL-23 than control subjects. More elevated IL-23 levels were shown in the subgroup analysis in females and in patients of ≥40 years of age. Furthermore, an association study was conducted with the IL-23 gene polymorphism rs11171806. The frequencies of rs11171806 alleles in Shanghai cohorts were markedly different between GD patients (G 95.7% and A 4.3%) and normal subjects (G 97.7% and A 2.3%) [P=2.6x10 -3, OR=1.93 (95% CI: 1.25–2.97)], and in Xiamen cohorts, the proportion of subjects with the A allele of rs11171806 was elevated and similar in GD patients and controls, [GD vs. control, 4.8% vs. 4.3%, OR=2.15 (95% CI: 1.23–3.79), Pallele=6.3x10 -3]. These findings suggested that in Han Chinese population the IL-23A gene can be considered a genetic risk marker (43).

4.5 TNF-α

A meta-analysis evaluated rs1800629 and rs361525 in TNF-α gene in case-control studies in order to clarify whether TNF-α can affect the susceptibility to GD (44). Ten case–control studies were included, with 2790 GD patients and 3472 normal controls. The rs1800629

polymorphism was significantly associated with GD, in the homozygous model (AA compared with GG: OR=1.97, 95% CI=1.27–3.06, P=0.002) and recessive model (AA compared with GA + GG: OR=1.62, 95% CI=1.04–2.50, P=0.03). After ethnicity stratification, in European population, GD susceptibility was significantly reported in all genetic models, whereas no significant association was shown in Asian population. A significant association of the SNP rs361525 with GD was not demonstrated. The data indicated that a raised risk of GD was associated with the promoter SNP rs1800629 in the TNF-α gene, especially in the European population (44).

4.6 IL-6

A meta-analysis evaluated the association between IL-6 -174 G/C polymorphism and GD, including 4 case-control studies with 554 GD patients and 1201 healthy subjects. In the combined analysis, the IL-6 -174 G/C polymorphism was significantly associated with the risk for GD, in dominant model (OR=1.39, 95% CI: 1.07-1.80), recessive model (OR=2.75, 95% CI: 1.01-7.55) and homozygote contrast model (OR=3.25, 95% CI: 1.1-9.58) (45).

To investigate the role of IL-6 gene methylation in AITD, the methylation levels of -666, -664, -610, -491 and -426 CpG sites in the IL-6 gene were evaluated in 29 HT patients, 31 GD patients and 16 healthy volunteers. Patients with intractable GD showed lower methylation levels at the -664 and -666 CpG sites than patients with GD in remission, suggesting a relation between the IL-6 gene methylation and the intractability of GD (46).

4.7 CCL21

CCL21 is important in several autoimmune diseases, but its role in GD is not clear. A study detected CCL21 levels in GD and examined the role of osteopontin (OPN; that stimulates the secretion of chemokines and proinflammatory cytokines, through NF-kB and MAPK) in

regulating CCL21 production (47). Fourty not previously treated, newly diagnosed GD, 12 GD patients with negative TRAb, 15 euthyroid GD patients, and 25 normal subjects were enrolled. CCL21 levels in plasma were higher in GD patients and normalized in those with negative TRAb; TRAb and plasma OPN correlated with CCL21. Recombinant OPN raised the expression of CCL21 in a time- and dose-dependent way. These findings pointed out a clinical correlation between GD and plasma CCL21, and that CCL21 could be considered a new marker in GD and a possible target to treat GD patients with positive TRAb (47).

5. Diagnosis

The diagnosis of hyperthyroidism is established according to specific clinical features and biochemical alterations. If pathognomonic features (such as dermopathy or ophthalmopathy) and a diffuse goiter are not present, radionuclide scanning permits to confirm the diagnosis, and to distinguish GD from other causes of thyrotoxicosis, such as the use of radioiodine uptake measurement. The measurement of TRAb, that has 99% sensitivity and specificity for GD, is not mandatory. TRAb assay is also useful in diagnosing GD in patients with concomitant nodular goiter (15).

In GO, when the cause of ocular manifestations is not clear, computed tomography or magnetic resonance imaging of the orbit is useful, and they permit to distinguish fat expansion from extraocular muscle enlargement. In particular, in presence of asymmetric proptosis, ruling out orbital tumor and arteriovenous malformation is determinant (15).

Other imaging techniques, such as orbital ultrasonography, gallium scanning, scintigraphy with radiolabeled octreotide, and thermal imaging, may be useful to define better the orbital disease (48).

6. Therapy

6.1 Hyperthyroidism

Antithyroid drugs are the first-line therapy for GD in Europe (49) and are increasingly preferred compared to radioiodine in North America. Ablative therapy, either from radioactive iodine or surgical thyroidectomy, can cause hypothyroidism and leads to lifelong thyroid hormone replacement (15). MMI, carbimazole (that is transformed to MMI and is not available in the United States), and propylthiouracil (PTU) inhibit TPO, blocking thyroid hormone synthesis. PTU also blocks extrathyroidal deiodination of T4 to T3. In Europe and North America, MMI is preferred for initial therapy thanks to its favorable side-effect profile (49). Both MMI and PTU are associated with an elevated risk of recurrence once stopped the therapy (15). Even going on with the therapy for more than 18 months or combining antithyroid drugs and levothyroxine, the recurrence rate is not furtherly decreased (15). Some experts recommend to monitor patients through liver-function tests and white-cell counts, before and during anti-thyroid drug treatment, even if this is not currently supported by consensus opinion (14).

Radioactive iodine has been used extensively in GD patients over the past decades, because it gives relief from symptoms of hyperthyroidism within weeks. To improve its effectiveness, antithyroid drugs can be suspended 3-7 days before and after radiotherapy. Radioiodine is not associated with a raised risk of cancer but it can cause or worsen ophthalmopathy. After radioiodine, it is necessary to monitor thyroid function all over the life, and if hypothyroidism occurs, it should be treated quickly (15,50-53).

Surgery is generally used in patients with a large goiter, women who wish to have pregnancy, and patients who do not want to receive anti-thyroid drugs or radioiodine (15). Thyroid hormone levels should be normal before surgical thyroidectomy, to reduce the risk of complications. The risks of hypothyroidism and recurrent hyperthyroidism are inversely related and are correlated to the residual tissue volume (54).

6.2 Antigen-specific immunotherapy

In GD, the existing treatments at present are not ideal for hyperthyroidism: anti-thyroid drugs lead to long-term remission in approximately 50% of patients, and radioiodine and surgery cause a durable medication-dependence (55). A more in-depth study of the pathogenesis of AITD is allowed by the knowledge of ATA and their measurement (26).

Antigen-specific immunotherapies aim to re-establish immune tolerance vs. the immune dominant epitopes implicated in autoimmunity, and are promising therapies for inflammatory, allergic, and autoimmune diseases, because they do not induce generalized immunosuppression (55).

Attempts to antigen-specific therapy have been recently done in AITD. Widespread immunosuppressive drugs can cause severe side effects, and antigen-specific therapy has been devised to establish tolerance in different autoimmune diseases (56).

A first phase I open label trial reported an attempt of immunotherapy in GD through the induction of T cell tolerance by TSH-R peptides (ATX-GD-59) (55). It is known that the pre-treatment with these peptides blunted the humoral and cell-mediated immune responses to TSH-R A-subunit adenovirus immunization in transgenic mice expressing the human HLA-DR3 molecule, while after the induction of hyperthyroidism injecting the TSH-R A-subunit protein was not effective. ATX-GD-59 was administered for a 18-week period to 12 hyperthyroid patients with mild-to-moderate untreated GD. Ten patients received ATX-GD-59 (10 doses), and about half of them had normal FT3 at the 18-week visit. Free thyroid hormones improved in 2 subjects, while 3 showed worsening thyrotoxicosis, at the end of the study. During the study, circulating TSH-R autoantibodies declined, correlating with different free thyroid hormones levels. The findings indicated that ATX-GD-59 is an effective treatment in GD (55).

6.3 Novel therapies in GO

High dose iv immunoglobulins (18), or corticosteroids (CS) reduce inflammation and orbital congestion in patients with active GO. At present, alternative immune-modulant therapies are under evaluation: IGF-1R and TSH-R (on B lymphocytes, fibroblasts, T lymphocytes), cytokines, and chemokines (implicated in autoimmunity), as possible targets of these novel therapies (57).

TNF-α has a crucial role in AITD, including GO (58). Etanercept derives from recombinant DNA, fusing the TNF receptor to the constant end of IgG1protein. It binds TNF-α, inhibiting its role in certain autoimmune diseases (59). Its use has been evaluated as possible therapy of 10 patients in the active phases of GO, with a remission in 6/10 (60).

IL-6 acts as a proinflammatory cytokine, secreted by T cells and macrophages to stimulate immune response. IL-6 and IL-6R are activated in GO patients with active disease, and elevated circulating levels of IL-6R have been shown (61). Tocilizumab is a humanized monoclonal antibody (mAb) against the IL-6R, approved for rheumatoid arthritis (RA) (62), Castleman's disease, and systemic juvenile idiopathic arthritis (63). Tocilizumab has been evaluated in a prospective nonrandomized study in 18 patients with GO (previously resistant to CS) (64). Proptosis ameliorated in 13 patients, diplopia in 7, and extraocular motility in 15. Tocilizumab was effective in the treatment of GO refractory to steroids, since no relapse of GO occurred (65).

A further recent paper evaluated 3 case reports (66) of GO patients, resistant to CS or with advanced diplopia, treated once-a-month with tocilizumab (iv; 8 mg/kg), reporting a significant amelioration in ocular symptoms (66).

Moreover, small TSH-R antagonist (NCGC00229600) led to a decreased production of hyaluronic acid in GO retro-orbital fibroblasts/adipocytes, reporting promising results (67).

Rituximab (RTX) is another proposed treatment for GO. It is a chimeric mAb against CD20, present on B cells surface. RTX induces B cells death, and for this reason it is indicated to treat disorders caused by dysfunctional B-lymphocytes, or overactive B-cells or elevated levels of B-lymphocytes (68). Food and Drug Administration approved RTX to treat RA, chronic lymphocytic leukemia, non-Hodgkin's lymphoma, and Wegener's granulomatosis, but it is also used off-label in other autoimmune disorders (69). In GO, RTX decreases the number of B lymphocytes, and the production of autoantibodies and cytokines; however only few papers hold up the therapy with RTX in GO patients (70), with conflicting results. A randomized, prospective, placebo-controlled study assessed the effect of RTX in 25 GO patients, administering 2 RTX (or 2 saline infusions) 2 weeks apart. A similar amelioration of the clinical activity score (CAS) was reported, supporting the ineffectiveness of RTX in GO (71). In a further double-blinded, randomized study, 32 patients received RTX or intravenous methylprednisolone (ivMP). Both treatments, especially RTX, reduced CAS. An improvement was shown in all RTX patients at 24 weeks vs. 69% after ivMP, demonstrating a higher effectiveness of RTX than ivMP in GO (72). A meta-analysis and systematic review evaluated four randomized controlled studies performed in 293 GO patients (treated with glucocorticoids or RTX, or saline). At 24 weeks CAS decreased significantly by RTX vs. controls, and a remarkable proptosis reduction, even if not significant, was observed (73).

In a study, 219 patients with Graves' ophthalmopathy received pulse MP, followed by oral steroids and/or orbital radiotherapy, and 15 patients with active disease ultimately received a total dose of 100–400 mg RTX doses. Low-dose RTX showed sustained anti-inflammatory action in most patients with active GO resistant to conventional therapies (74).

Another study confirmed these findings. Twelve GO patients in the active phase of the disease were administered with a RTX infusion (100 mg), that was effective in decreasing disease activity, allowing a reduced dose of systemic steroids (75).

Another study reported 14 patients with active and moderate-to-severe GO (eleven of whom resistant to CS) treated with RTX (iv; 1000 mg twice at a 2-week interval) (76). A reduced amelioration in CAS was observed (P=0.002). About 50% of patients had a disease inactivation. At 12 weeks, inactivation of GO was in 28.6% of patients, and CAS ameliorated in 14.3%. At 24 weeks, proptosis and total eye score ameliorated in 33% and in 28.6% of patients, respectively. The reported findings indicated that RTX is safe and well-tolerated in GO (76).

Another promising treatment for GO is teprotumumab (RV 001, R1507), a human monoclonal anti-IGF-1R blocking antibody. In orbital fibroblasts, TSH-R and IGF-1R form a physical and functional complex. A subset of these fibroblasts derives from infiltrating CD34(+) fibrocytes. Teprotumumab is able to reduce *in vitro* fibrocyte IGF-1R and TSH-R, and the TSH induction of IL-6 and IL-8 mRNA and protein (77).

In order to investigate the efficacy of teprotumumab, in patients with active, moderate-to-severe GO, a randomized, multicenter, placebo-controlled, double-masked trial was performed (78). Eighty-eight patients received placebo, or the active drug (8 infusions). The response in the study eye (at week 24, a decrease in CAS of 2 points or more, and a decrease in proptosis of 2 mm or more) was the primary end point. At week 24, a response was shown in 29/42 patients treated with teprotumumab (69%), and 9/45 patients receiving placebo (20%) ($P<0.001$). At week 6, 18/42 patients trated with teprotumumab (43%) and 2/45 patients receiving placebo (4%) had a response ($P<0.001$). In patients with diabetes, hyperglycemia was the only drug-related adverse event, and medication for diabetes was adjusted. The above reported data, in patients with active ophthalmopathy, showed that teprotumumab was more effective than placebo in reducing proptosis and CAS (78).

7. Conclusion

GD is clinically characterized by thyrotoxicosis, and by the presence of serum ATA and thyroidal infiltration of autoreactive lymphocytes. In GD, autoimmune reaction induces the production of TSAb by B-cell clones infiltrating the thyroid. These antibodies are determinant in the pathogenesis of GD and its extra-thyroidal manifestations (i.e. GO and pretibial myxedema (PTM)/Graves' dermopathy). The GD risk factors include genetic predisposition, and interactions between endogenous and environmental factors (79).

Th1 immune response prevails in the immunopathogenesis of GD and GO, during which Th1 chemokines, and the (C-X-C)R3 receptor, play a key role. In GD, recruited Th1 lymphocytes lead to an increased IFN-γ and TNF-α production, that stimulates Th1 chemokines secretion from thyroid cells, reiterating the autoimmune process. Elevated serum Th1 chemokines levels are associated with the active phases of GD. Methimazole, and corticosteroids are able to modulate these chemokines.

In GD, the existing treatments at present are not ideal for hyperthyroidism: anti-thyroid drugs lead to long-term remission in only 50% of patients, and radioiodine and surgery cause hypothyroidism. In GD, antigen-specific therapy has been recently published, with the induction of T cell tolerance via an immunization by TSH-R peptides.

RTX (a chimeric mAb against CD20) and drugs targeting cytokines [i.e. anti-IL-6 (Tocilizumab), and anti-TNF-α (Etanercept)] have been evaluated, with encouraging findings in GO. Teprotumumab (a human monoclonal anti-IGF-1R blocking antibody) showed to be very effective in GO patients.

Further researches are necessary to identify novel specific and effective therapies targeting GD, or GO.

Conflict of Interest

The Authors have nothing to declare.

Role of the funding source

The Authors have nothing to declare.

Summary

Different factors are implicated in the GD pathogenesis, i.e. genetic factors, interactions between endogenous and environmental factors, and immune system dysregulation. TRAb are determinant in the pathogenesis of GD and its extra-thyroidal manifestations (i.e. GO and pretibial myxedema/Graves' dermopathy).

In the immunopathogenesis of GD and GO, a Th1 immune response prevails; in GD, recruited Th1 lymphocytes lead to an increased IFN-γ and TNF-α production, that stimulates Th1 chemokines secretion from thyroid cells, reiterating the autoimmune process. Elevated serum Th1 chemokines levels are associated with the active phases of GD. Methimazole, and corticosteroids are able to modulate these chemokines.

In GD, the existing treatments are not ideal for hyperthyroidism (long-term remission with anti-thyroid-drugs only in 50% of patients; while radioiodine and surgery cause hypothyroidism). Antigen-specific therapy has been recently published in GD, with the induction of T cell tolerance via an immunization by TSH-R peptides.

Encouraging findings arose with RTX (a chimeric mAb against CD20) and drugs targeting cytokines [i.e. anti-IL-6 (Tocilizumab), and anti-TNF-α (Etanercept)] for the treatment of GO. Teprotumumab (a human monoclonal anti-IGF-1R blocking antibody) showed to be very effective in GO patients.

Further researches are necessary to identify novel specific and effective therapies targeting GD, or GO.

Practice Points

-GD is characterized by thyrotoxicosis, due to the presence of circulating thyroid stimulating antibodies (TSAb), that are determinant also in the pathogenesis of its extrathyroidal manifestations [GO, pretibial myxedema].

- Th1 immune response prevails in the immunopathogenesis of GD and GO, during the active phase.

-In GD, the existing treatments at present are not ideal for hyperthyroidism: anti-thyroid drugs lead to long-term remission in only 50% of patients, and radioiodine and surgery cause hypothyroidism.

Research Agenda

-In GD, antigen-specific therapy with the induction of T cell tolerance via an immunization by TSH-R peptides, has been recently reported.

-RTX (a chimeric mAb against CD20) has been evaluated, with encouraging findings in GO.

-In GO, drugs targeting cytokines [i.e. anti-IL-6 (Tocilizumab), and anti-TNF-α (Etanercept)] have been evaluated, with encouraging findings.

-Teprotumumab (a human monoclonal anti-IGF-1R blocking antibody) showed to be very effective in GO patients.

- Further researches are necessary to identify novel specific and effective therapies targeting GD, or GO.

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