

# Pathogenesis of thyroid eye disease: review and update on molecular mechanisms

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## ABSTRACT

Orbital changes in thyroid orbitopathy (TO) result from de novo adipogenesis, hyaluronan synthesis, interstitial oedema and enlargement of extraocular muscles. Cellular immunity, with predominantly CD4+ T cells expressing Th1 cytokines, and overexpression of macrophage-derived cytokines, perpetuate orbital inflammation. Orbital fibroblasts appear to be the major effector cells. Orbital fibroblasts express both thyrotropin receptor (TSHR) and insulin-like growth factor-1 receptor (IGF-1R) at higher levels than normal fibroblasts. TSHR expression increases in adipogenesis; TSHR agonism enhances hyaluronan production. IGF-1R stimulation leads to adipogenesis, hyaluronan synthesis and production of the chemokines, interleukin (IL)-16 and Regulated on Activation, Normal T Cell Expression and Secreted, which facilitate lymphocyte trafficking into the orbit. Immune activation uses a specific CD40:CD154 molecular bridge to activate orbital fibroblasts, which secrete pro-inflammatory cytokines including IL-1 $\beta$ , IL-1 $\alpha$ , IL-6, IL-8, macrophage chemoattractant protein-1 and transforming growth factor- $\beta$ , to perpetuate orbital inflammation. Molecular pathways including adenylyl cyclase/cyclic adenosine monophosphate, phosphoinositide 3 kinase/AKT/mammalian target of rapamycin, mitogen-activated protein kinase are involved in TO. The emergence of a TO animal model and a new generation of TSHR antibody assays increasingly point towards TSHR as the primary autoantigen for extrathyroidal orbital involvement. Oxidative stress in TO resulting from imbalances of the oxidation-reduction state provides a framework of understanding for smoking prevention, achieving euthyroidism and the use of antioxidants such as selenium. Progress has been made in the understanding of the pathogenesis of TO, which should advance development of novel therapies targeting cellular immunity, specifically the CD40:CD40 ligand interaction, antibody-producing B cells, cytokines, TSHR and IGF-1R and its signalling pathways. Further studies in signalling networks and molecular triggers leading to burnout of TO will further our understanding of TO.

## INTRODUCTION

Thyroid orbitopathy (TO) is an autoimmune inflammatory disorder involving the orbit. Ninety per cent of patients with TO have Graves' disease (GD) and are hyperthyroid, 5% are hypothyroid and another 5% are euthyroid.<sup>1 2</sup> Many patients with TO develop eye symptoms within the first 18 months of autoimmune thyroid disease, with 13% of patients presenting beyond 2 years, and 3% preceding the diagnosis of GD by >12 months.<sup>3 4</sup>

In the USA, the age-adjusted incidence rate of TO was 16/100 000 population/year in females and

2.9 cases/100 000/year in males.<sup>1</sup> When only moderate-to-severe TO is considered, the incidence rate reduces to 16.1 cases/million/year, regardless of salt iodination.<sup>5</sup> Predicted prevalence rates of TO are stable across different countries, ranging from 0.1% to 0.3%.<sup>6</sup> In newly diagnosed GD, 20% have mild and inactive TO, 5.8% present with moderate to severe, active TO and 0.3% develop compressive optic neuropathy in a non-tertiary setting.<sup>7</sup> The evidence is strong for the association of smoking and TO; smokers have an increased risk for TO, and severity of TO correlates with smoking in a dose-dependent manner.<sup>8 9-11</sup> In addition, uncontrolled hypothyroidism and hyperthyroidism, and radioactive iodine therapy have been associated with development of TO in clinical studies.<sup>12-14</sup> Cessation of smoking, achievement of euthyroidism and prophylactic oral prednisolone prior to radioactive iodine therapy in at-risk patients form important preventive steps to control these modifiable risk factors for TO.<sup>15-17</sup>

Recent advances in transcriptomics and proteomics have brought new insights into the molecular basis of TO. These discoveries have led to the emerging use of monoclonal antibodies and will undoubtedly eventually lead to more specific therapies for this challenging condition. This review explores the underlying molecular mechanisms of TO, highlighting the basis for emergent prevention and treatment options.

## PATHOLOGICAL CHANGES IN TO

Pathological changes of TO in the orbit appear to involve both the extraocular muscles and the orbital fat compartments, with CT indicating most patients have a mixture of both extraocular muscle enlargement and orbital fat expansion.<sup>18</sup> Proptosis is due to expansion of orbital tissue within the unyielding confines of the bony orbit. The consequent increase in orbital pressure can also lead to venous outflow congestion and chronic periorbital oedema.<sup>19</sup>

Histological examination of affected extraocular muscles shows extraocular muscle enlargement is due to deposition of glycosaminoglycan (GAG), predominantly hyaluronan (HA) within the muscles' endomysial space.<sup>20</sup> Total orbital GAG in TO is markedly elevated with significant increase in chondroitin sulfate and HA; correspondingly 24 h urinary total GAG, dermatan sulfate and HA are also elevated in TO compared with normal controls.<sup>21 22</sup> HA from orbital cells is primarily >500 000 Da high-molecular-weight polymers.<sup>23</sup> As HA is highly anionic, intense water binding leads to pronounced orbital interstitial oedema and



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extraocular muscle expansion without disruption of muscle fibres.<sup>20 21 23</sup>

Furthermore, histology of extraocular muscles shows diffuse and focal lymphocytic infiltrates and fibrosis, whereas orbital fat and connective tissue contains few infiltrating cells. The majority of mononuclear cells are T cells, along with a few B cells, macrophages and mast cells in the intercellular space.<sup>24 25</sup> Macrophages, monocytes and mast cells are also located in the perivascular interstitial space and in between fibroblast cells with co-localisation of platelet-derived growth factors in the orbital tissue.<sup>26 27</sup>

### EFFECTOR CELL IN TO

Current evidence suggests the orbital fibroblast is the key effector cell in TO.<sup>28</sup> Not only do orbital fibroblasts proliferate and differentiate into myofibroblasts and adipocytes, they produce GAG in excess, undergo adipogenesis and actively interact with mononuclear cells, produce chemoattractants and cytokines, which ensure perpetuation of orbital inflammation.<sup>26 29–31</sup> Most of our understanding of orbital fibroblasts in the pathophysiology of TO is derived from *in vitro* culture studies. Orbital fibroblasts and pre-adipocyte cultures when subjected to differentiation medium underwent adipogenesis with increased peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) transcripts and lipoprotein lipase (LPL) expression, accompanied by increased HA production and hyaluronic acid synthase 2 (HAS2) mRNA transcripts.<sup>32</sup> Interleukin (IL)-1 $\beta$  and leukoregulin stimulate a marked increase in HA secretion in TO orbital fibroblasts.<sup>23 33 34</sup> Activated orbital fibroblasts from TO showed a robust response to pro-inflammatory cytokines compared with normal controls and secrete higher levels of pro-inflammatory cytokines including IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, macrophage chemoattractant protein-1 (MCP-1), transforming growth factor (TGF)- $\beta$  when stimulated by cytokines and growth factors.<sup>35–38</sup>

Heterogenous presentations of TO could be due to cellular divergence of orbital fibroblasts within the orbit.<sup>31</sup> The fibroblast populations in the orbit are phenotypically heterogeneous and differ with regard to surface glycoprotein, production of pro-inflammatory cytokines and cell surface receptors.<sup>36 39 40</sup> The perimysial orbital fibroblasts uniformly express Thy-1, whereas adipose tissue orbital fibroblasts show bimodal distribution of both Thy-1-positive and Thy-1-negative cells.<sup>39 40</sup> Both Thy-1-positive and Thy-1-negative fibroblasts express high levels of PPAR- $\gamma$  but only the Thy-1-negative adipose orbital fibroblasts differentiate and accumulate lipid droplets.<sup>40</sup> On the other hand, only Thy-1-positive orbital fibroblasts can differentiate into myofibroblasts on stimulation with TGF- $\beta$ .<sup>41</sup>

The innate depot differences in fibroblasts may also explain the predilection for orbital and pretibial extra-thyroidal involvement in GD. Adipogenesis and HA synthesis in orbital pre-adipocytes and fibroblasts is site specific, occurring in both TO and normal controls.<sup>32</sup> Regional differences exist in basal PPAR- $\gamma$  expression and responses of human pre-adipocytes to PPAR- $\gamma$  and retinoid X receptor  $\alpha$  agonists.<sup>42</sup> Orbital fibroblasts also express considerably higher IL-6 and IL-6 receptor, and prostaglandin E2 (PGE2) than dermal fibroblasts when induced by IL-1 $\beta$  and leukoregulin, respectively.<sup>43 44</sup>

### MOLECULAR MECHANISMS UNDERLYING TO

The molecular mechanisms whereby recruitment of immune cells into the orbit, the molecular bridge between immune cells and orbital fibroblasts, molecular pathways leading to proliferation and differentiation of orbital fibroblast, secretion of HA,

adipogenesis and perpetuation of orbital inflammation are now better understood (figure 1).

### Cellular immunity

T cell infiltrates in TO orbital tissues are predominantly CD4+, with some studies suggesting presence of both CD8+ and CD4+ T cells.<sup>45–48</sup> Th1-like cytokine profile predominates in TO retrobulbar tissue.<sup>45 48</sup> Th1-like cytokine expression profile consisting of interferon (IFN)- $\gamma$ , tumour necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$  and IL-6 has been detected mainly in TO extraocular muscles, whereas IL-4 and IL-10, Th2-type cytokines were detected predominantly in orbital fat.<sup>38</sup> Predominance of T cell subsets is also disease duration dependent, with Th1 cells dominating in the active phase of TO, shifting towards Th2 cells in the late phase.<sup>49</sup>

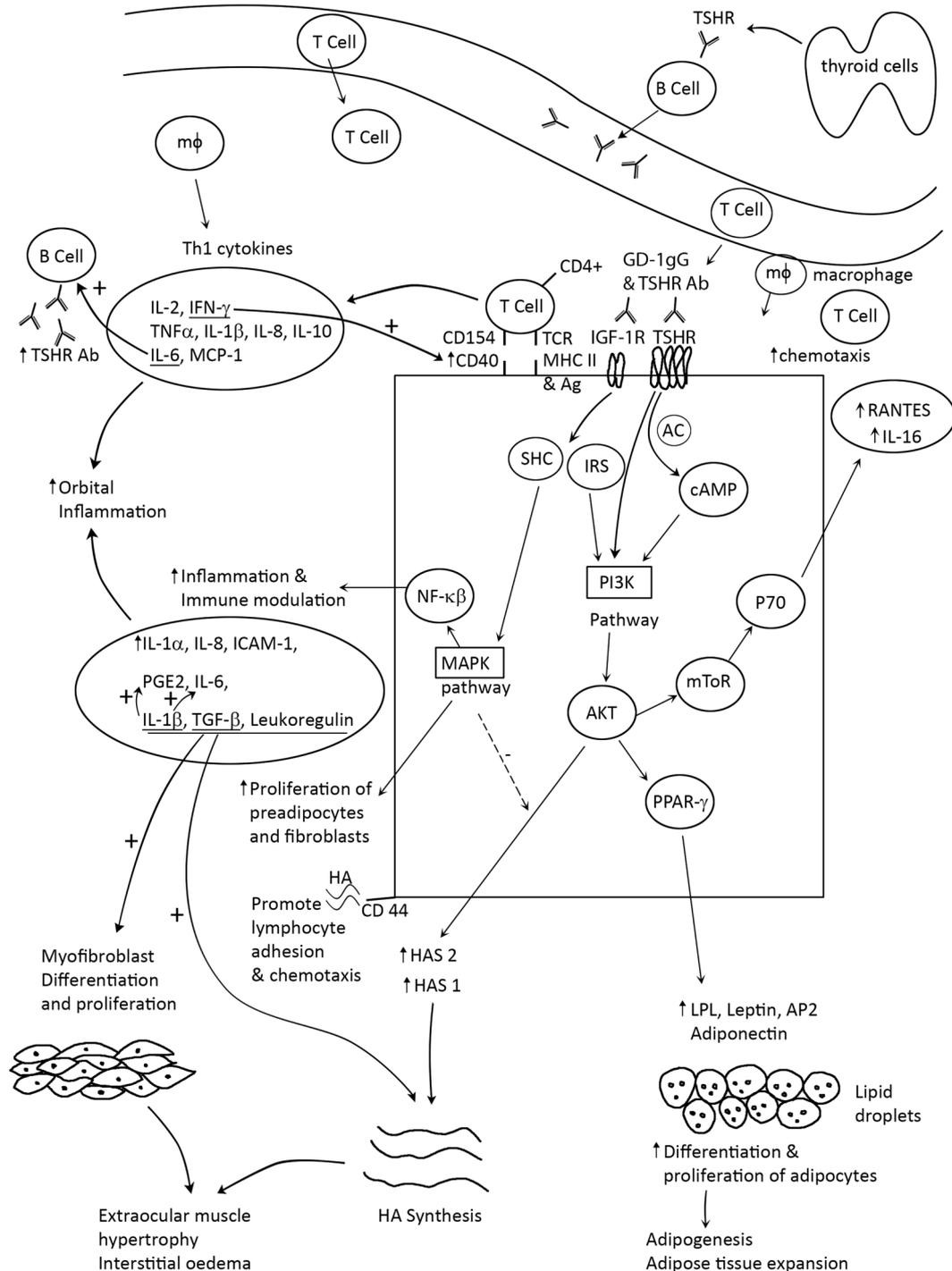
Proliferation of orbital fibroblasts is activated by interaction of autoantigens on the fibroblasts with T cells that involve contact of T cell receptor with Major histocompatibility complex class II molecule and CD40:CD154 signalling.<sup>30</sup> Co-culture of orbital fibroblasts with autologous T cells stimulates production of MHC II molecule and proliferation of orbital fibroblasts in a dose-dependent manner; blocking antibodies to MHC II, CD40 and CD40 ligand (CD154) completely inhibit proliferation of orbital fibroblasts.<sup>30</sup> CD40 expression is upregulated in orbital fibroblasts by IFN- $\gamma$  mediated through Jak2.<sup>36</sup> Ligation of CD40 with CD154 induces increased secretion of intercellular adhesion molecule-1 (ICAM-1),<sup>50</sup> nuclear translocation of nuclear factor- $\kappa$ B (NF- $\kappa$ B),<sup>51</sup> IL-6, IL-8 and MCP-1 in TO orbital fibroblasts compared with normal controls.<sup>36</sup> In addition, CD40 upregulates IL-1 $\alpha$  secretion, HA and PGE2 synthesis.<sup>52</sup> The molecular signalling triggered by CD40:CD154 ligation involve all three mitogen-activated protein kinase (MAPK) pathways, p38, ERK1/2 and JNK, which mediate cellular activities such as gene expression, cellular proliferation, differentiation and apoptosis. ICAM expression is predominantly P38 MAPK and NF- $\kappa$ B dependent, whereas ERK1/2 and JNK also activate the NF- $\kappa$ B pathway, a transcription factor pathway that regulates genes involved in immune and inflammatory responses.<sup>50</sup>

### Role of cytokines

Study of the cytokine profile in orbital adipose tissue in TO and normal individuals shows overexpression of IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$ , IL-6 and IL-10, which are macrophage-derived and IL-8. IL-1 $\beta$  is expressed the most differentially.<sup>37</sup> Similarly, patients with active TO have higher IL-1 $\beta$ , IL-6, IL-8 and IL-10 compared with inactive TO.<sup>53</sup> Orbital fibroblasts from TO when stimulated by IL-1 $\beta$  upregulate secretion of pro-inflammatory cytokines IL-6 and IL-8, PGE2, IL-6R and T cell chemoattractants, IL-16 and Regulated on Activation, Normal T Cell Expression and Secreted (RANTES), which recruit T cells into the orbit.<sup>39 43 44 54</sup> IFN- $\gamma$  upregulates CD40 expression on orbital fibroblasts and fibrocytes.<sup>55</sup> IL-6 increases the expression of thyrotropin receptor (TSHR) in orbital fibroblast pre-adipocytes and promotes B cell differentiation and immunoglobulin production.<sup>56 57</sup> IL-1 $\beta$  uses p38 and ERK1/2 MAPK pathways to induce IL-6 gene expression.<sup>43</sup> Immunoglobulin G from patients with GD substantially upregulates RANTES and IL-16, AKT/FRAP/mammalian target of rapamycin (mTOR)/p70 pathway is implicated in the induction of IL-16.<sup>54</sup>

### HA synthesis

IL-1 $\beta$ , leukoregulin, CD154, TGF- $\beta$ 1 and platelet-derived growth factor (PDGF) are all involved in stimulating HA synthesis, likely via receptor and ligand binding on the orbital



**Figure 1** Model of pathogenesis of thyroid orbitopathy (TO). T cell interacts with orbital fibroblast via CD40:CD154 ligation, and interaction of Major histocompatibility complex class II (MHC II), autoantigen and T cell receptor activate orbital fibroblast with increase secretion of intercellular adhesion molecule-1 (ICAM-1), nuclear translocation of nuclear factor (NF)- $\kappa$ B, interleukin (IL)-1, IL-6, IL-8, macrophage chemoattractant and prostaglandin E2 (PGE2) secretion. Cytokines showed Th1 dominance with increase IL-1 $\beta$ , interferon (IFN)- $\gamma$ , tumour necrosis factor (TNF)- $\alpha$  and IL-6. IFN- $\gamma$  increases CD40 expression, IL-6 modulates B cell immunoglobulin (Ig) secretion. Orbital fibroblast upregulates pro-inflammatory cytokines IL-1 $\beta$ , transforming growth factor (TGF)- $\beta$ , leukoregulin that perpetuate orbital inflammation and increase hyaluronan (HA) synthesis. TGF- $\beta$  induces myofibroblast proliferation and differentiation and promotes lymphocyte adhesion and chemotaxis by CD44 and HA interaction. IgG from Graves' disease (GD) and insulin-like growth factor-1 (IGF-1) upregulates secretion of Regulated on Activation, Normal T Cell Expression and Secreted (RANTES) and IL-16, which increase T cell migration into the orbit; the AKT/mammalian target of rapamycin (mTOR)/P70 pathway seems involve in IL-16 upregulation. Activating thyrotropin receptor (TSHR) increases hyaluronan synthase (HAS) via adenylyl cyclase/cyclic adenosine monophosphate (cAMP) and AKT/phosphoinositide 3 kinase (PI3K) pathway. IGF-1 can also induce HAS and HA synthesis, the effect is unmasked by mitogen activated protein kinase (MAPK) inhibitor. Both TSHR and IGF-1R activate PI3K/AKT pathway to upregulate peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) expression, differentiation and proliferation of adipocytes and enhance adipogenesis. IGF-1R uses Src homology 2 domain-containing (SHC)/insulin receptor substrate (IRS)/MAPK signalling to increase proliferation of pre-adipocytes. Switching off proximal SHC signalling on MAPK in turn permit differentiation of pre-adipocyte to adipocytes and enhance adipogenesis.

fibroblast.<sup>26 34 35 58</sup> Orbital fibroblast surface receptors for TSHR and insulin-like growth factor-1 receptor (IGF-1R) both appear to stimulate HA synthesis. TSHR activation alone is sufficient to upregulate expression of HAS1 and HAS2 and HA production via cyclic adenosine monophosphate (cAMP) and AKT/phosphoinositide 3 kinase (PI3K) signalling with upregulation of HA production.<sup>23 59 60</sup> On the other hand, both immunoglobulin G (IgG) from GD and IGF-1 stimulate an equivalent and substantial increase in HA synthesis in orbital fibroblasts, suggesting alternative IGF-1R pathways are also involved in HA synthesis.<sup>61 62</sup> However, the effect of IGF-1 on HA synthesis appears indirect as IGF-1 alone does not increase HAS2 transcription. The stimulatory effect of IGF-1 on HAS transcription is unmasked by MAPK kinase inhibitor but not mTOR or PI3K inhibitors in orbital fibroblasts.<sup>32</sup>

IL-1 $\beta$  is a potent stimulator for GAG synthesis. Increased secretion of HA in TO orbital fibroblasts by IL-1 $\beta$  is due to predominant induction of HAS2, and to a lesser extent, HAS3. The effects of HAS mRNA induction by IL-1 $\beta$  can be inhibited by glucocorticoids.<sup>33</sup> PDGF $\beta$  and TGF are growth factors that are significantly increased in TO orbital tissues. They induce orbital fibroblast proliferation and stimulate HAS1 and HAS2 expression in TO orbital fibroblasts.<sup>35 58</sup> TGF- $\beta$  acts via the Smad pathway.<sup>35</sup> TGF- $\beta$ -treated orbital fibroblasts also bind activated human T cells through HA-CD44 interaction, thus promoting lymphocyte chemotaxis and adhesion to pro-inflammatory sites.<sup>58</sup> Addition of PPAR- $\gamma$  ligands, on the other hand, inhibits TGF- $\beta$ -induced HAS1 and HAS2 expression and attenuate HA synthesis independent of the PPAR- $\gamma$  pathway.<sup>58</sup>

### Adipogenesis

De novo adipogenesis is enhanced in TO as evidenced by increased expression of adipocyte-specific genes leptin, adiponectin, fatty acid synthase, adipocyte fatty acid binding protein (AP2) and PPAR- $\gamma$  mRNA in TO-affected adipose tissue compared with normal orbital tissue.<sup>63 64</sup> Microarray studies provide further evidence that adipocyte-related intermediate early genes, including CYR61, are overexpressed in active TO.<sup>65</sup>

PPAR- $\gamma$  is a potent stimulator for adipogenesis in TO, evident by increased expression of PPAR- $\gamma$  in active TO adipose tissue compared with normal controls.<sup>66</sup> PPAR- $\gamma$  agonist, rosiglitazone, increases TSHR expression, PPAR- $\gamma$  mRNA and cAMP levels by 2.6–4.7-fold, resulting in adipogenesis in TO orbital fibroblasts both by proliferation and differentiation of adipocytes.<sup>67</sup>

Signalling for adipogenesis has been shown to involve both TSHR and IGF-1R. It appears both TSHR and IGF-1R share the same intracellular AKT/PI3K signalling to affect adipogenesis. The close relationship of TSHR and IGF-1R in triggering adipogenesis in TO perhaps could be explained by co-localisation of these two receptors on orbital fibroblasts.<sup>68</sup> Stimulatory TSHR antibody increases phosphorylated AKT protein, cAMP levels and enhanced adipogenesis via the PI3K signalling cascade.<sup>69</sup> On the other hand, IGF-1 mediates proliferation and differentiation of human and murine 3T3-L1 pre-adipocytes into adipocytes.<sup>70 71</sup> IGF-1 mediates its effect by binding to IGF-1R and induces phosphorylation of Src homology 2 domain-containing protein (Shc) and insulin receptor substrate (IRS) and downstream AKT/PI3K pathway.<sup>71 72</sup> IGF-1 uses Shc/IRS-1 to activate MAPK/ERK signalling in proliferating 3T3-L1 pre-adipocytes. Inhibiting MAPK by Shc proximal signalling switches off proliferation of pre-adipocytes and in turn permits differentiation into adipocytes with increased expression of PPAR- $\gamma$ , LPL and AP2.<sup>73</sup>

### AUTOANTIGENS IN TO TSH receptor

Breaking of self-tolerance to TSHR on thyroid epithelial cells, resulting in TSHR stimulating antibodies inducing thyrotoxicosis, is well established in GD.<sup>74 75</sup> TSHR signals mainly by two G-protein mediated pathways: the adenylyl cyclase/cAMP pathway and the PI3K/AKT/mTOR pathway.<sup>76</sup> Evidence from the temporal correlation of TO and GD, emerging TO animal models and correlation of disease activity and TSHR antibody increasingly point towards TSHR as the primary autoantigen in TO.

The observation that onset of TO is frequently within 18 months of diagnosis of GD<sup>4</sup> raised early on the concept that the two clinical entities are triggered by a common autoantigen. The first evidence of TSHR as an autoantigen came from identifying TSHR expression in retro-orbital tissue in cultured orbital fibroblast from patients with TO by PCR and liquid hybridisation.<sup>77–79</sup> Of note, the level of TSHR expression on orbital fibroblast is only of low abundance compared with thyrocytes but increases during adipogenesis and in active TO.<sup>67 80</sup>

With improvement of TSHR assays, both thyroid binding inhibiting Ig (TBI) and thyroid stimulating Ig (TSI) TSHR titres are shown to be highly and significantly correlated with activity and severity of TO, thus inferring TSHR antigen is pathogenic in TO.<sup>81</sup> The newer chimeric TSHR and cAMP response element-dependent luciferase MC4/TSI assay has higher sensitivity (97%) and specificity (89%) than the current TBI assay (77% and 43%, respectively) in TO.<sup>82</sup> The new MC4/TSI assay correlates strongly with clinical activity and clinical severity scores in both adults and children.<sup>82 83</sup> In the uncommon patients with euthyroid TO, TSHR antibody was highly detectable at 93.8% using third-generation TSI assay and 81.3% in second-generation TBI assay in comparison to the low TSHR positivity (18.8%) in first-generation assays.<sup>84</sup> Therefore, insensitivity of earlier TSHR assays seems likely to explain the seemingly poor correlation of TSHR antibody with severity of TO seen in the past.

### IGF-1R

IGF-1R is a ubiquitous cellular surface heterotetrametric receptor involved in diverse cellular responses including modulation of apoptosis, enhancing cell survival, growth and cellular proliferation, cell motility and migration.<sup>68 85</sup> Evidence suggests IGF1/IGF-1R is involved in the pathogenesis of TO, but the autoantigenic role of IGF-1R remains controversial. IGF-1R regulates lymphocyte trafficking in the orbit, HA synthesis, adipogenesis and defines T-lymphocyte and B-lymphocyte phenotypes and function.<sup>86</sup> IGF-1R levels are three fold higher on TO compared with control fibroblasts.<sup>68</sup> IgG from patients with GD induces IL-16 and RANTES secretion mediating T cell migration.<sup>54</sup> These effects are shown to be induced by IGF-1 and IGF-1R-specific ligand, Des(1–3) IGF-1 analogue, but not TSH.<sup>87</sup> Moreover, upregulation of IL-16, RANTES secretion and HA synthesis was restricted to GD orbital and dermal fibroblast and was not observed in normal control fibroblasts. Interfering with IGF1-R function completely abolished signalling induced by IgG from GD, hence implying IGF-1R is a self-antigen mediating T cell migration, lymphocytes infiltration and HA synthesis in TO.<sup>61 87</sup> A recent case-control microarray study also showed differentially expressed genes are dominated by IGF-1 signalling genes, with significant upregulation of *IGF-1*, IGF-1 signalling genes *SOCS3* and *SGK-1* (PDK/AKT signalling) and downregulation of *IRS2* and *IGFBP6* in TO.<sup>88</sup>

It is now clear that once an IGF-1R antibody assay became available that IGF-1R antibody is present in both patients with GD and healthy controls. The prevalence of IGF-1R antibody in patients with TO and healthy controls is similar (11% in normal and 14% in TO), and there is no correlation of clinical activity score or severity of TO with IGF-1R antibody level; elevated IGF-1R antibody levels in TO also remain stable over 2 years.<sup>89</sup> Furthermore, IGF-1R antibody binds IGF-1R and interferes with IGF1-dependent receptor activation and signalling; its effect is inhibitory on hepatocarcinoma and breast cancer cells proliferation.<sup>89</sup> Hence, these findings do not support IGF-1R as an autoantigen in TO. On the other hand, in an animal model, mice challenged with IGF-1 $\alpha$  plasmid produced strong IGF-1R antibody response, but did not induce hyperthyroidism or orbital changes.<sup>90</sup> Conversely injection of TSHR A subunit plasmid combined with electroporation induces hyperthyroidism, and both TSHR stimulating antibody and IGF-1R antibody.<sup>90</sup>

### EMERGING TO ANIMAL MODEL

Almost all animal models of GD use in vivo expression of TSHR either by transfected cells, plasmid or adenovirus. TSH subunit A seems to initiate the autoimmune response to TSHR.<sup>91</sup> Many animal models developed for GD develop hyperthyroidism but fail to show TO manifestations. One that did induce orbital pathology using a splenocyte adoptive transfer model with observed extraocular muscle oedema, accumulation of PAS-positive material, expansion of adipose tissue, dissociation of muscle fibres, lymphocyte and mast cell infiltration was not reproducible.<sup>91–93</sup>

A breakthrough in establishing a TO animal model was reported by Banga using TSHR A-subunit plasmid-immunised by muscle electroporation in BALB/c mice.<sup>94</sup> It showed orbital remodelling with bilateral interstitial inflammatory infiltrate in the extraocular muscle, infiltration of CD3+ T cells, F4/80+

macrophages and mast cell, orbital fibrosis, GAG deposition and corresponding MRI changes of orbital muscle hypertrophy. A few mice also showed predominantly expansion of retro-orbital fat, proptosis, chemosis and congested orbital vessels. This is by far the most representative animal model of TO. TSHR and a lower level of IGF-1R antibodies were both induced.<sup>94</sup> The less expected findings were predominance of TSH blocking antibodies, hypothyroid status and large inflammatory infiltrates around the optic nerve, which are not typical of GD. Nevertheless, these findings support the pathogenic role of TSHR in the development of TO and open the door for investigating pathogenesis and therapeutic drugs in an animal TO model. Interestingly using a similar protocol with a slight alteration of the electroporation regimen in an earlier study, TSHR plasmid induced a high frequency of hyperthyroidism (75%), TSHR stimulating antibodies, and in some animals, orbital connective tissue fibrosis.<sup>90</sup>

### OXIDATIVE STRESS AND TO

A state of oxidative stress has been described in GD and TO.<sup>95–96</sup> An increase in reactive oxygen species or reduced elimination of radicals by antioxidative enzymes will result in oxidative damage to cell membrane with lipid peroxidation and oxidative DNA damage, resulting in inflammation and loss of function.<sup>97</sup>

Both 8-hydroxy 2'-deoxyguanosine (8-OHdG) and malondialdehyde, as well as intracellular superoxide anion and hydrogen peroxide, were significantly elevated in TO orbital fibroblast compared with normal controls.<sup>98</sup> The 8-OHdG urinary levels correlate well with clinical activity score.<sup>99</sup> These findings suggest increased oxidative DNA damage and lipid peroxidation may have a role in the pathogenesis of TO. Increased oxidative stress is also noted in vivo, where lipid hydroperoxide, superoxide dismutase (SOD), glutathione reductase and glutathione peroxidase are significantly elevated in orbital fibroadipose tissue, while glutathione (antioxidant) is reduced compared with controls. Glutathione levels are strongly negatively correlated with the ophthalmopathy index.<sup>95</sup>

In hyperthyroid patients, achieving euthyroidism with methimazole results in all markers of oxidative stress being normalised in GD without orbitopathy, but not entirely in the TO group where oxidative stress indices remain significantly different from normal controls.<sup>96</sup> Similarly, oxidative stress marked by tert-butyl hydroperoxide initiated chemiluminescence remains high after radioactive iodine treatment.<sup>100</sup> Treatment with oxygen radical scavengers and antithyroid drugs reduce hydrogen peroxide-induced and, to a lesser degree, heat-induced 72 kDa heat shock protein (HSP72). HSP72 is a cytosolic protein inducible by heat shock and ischaemia, and its expression is increased in autoimmune thyroid disease.<sup>101–102</sup>

Reactive oxygen species (superoxide anions and hydrogen peroxide) induce pro-inflammatory cytokines production (IL-1 $\beta$ , TGF- $\beta$ 1) and stimulate orbital fibroblast proliferation in a dose-dependent manner; the proliferative effect can be inhibited by multiple antioxidants, methimazole but not propylthiouracil.<sup>103–104</sup> Free radicals are also involved in IL-1 $\beta$ -induced GAG production in TO orbital fibroblasts. IL-1 $\beta$  increases free radical production in both normal and TO orbital fibroblast, and stimulates SOD activity in TO orbital fibroblasts. Furthermore, reducing oxygen-free radicals with SOD and catalase partially blocked IL-1 $\beta$ -induced GAG production.<sup>105</sup> Nicotinamide reverses cellular injury in the orbit by inhibiting cytokine-induced activation in TO orbital fibroblasts.<sup>106</sup>

**Table 1** Novel and potential immunotherapies in clinical and preclinical trials for TO

Class of drugs	Mechanism of action	Example
CD20 monoclonal antibody	Deplete B cells and precursor by recognising surface CD20 marker	Rituximab <sup>110–112</sup>
IL-6 receptor monoclonal antibody	Binding to soluble and membrane bound IL-6 receptor and inhibit pro-inflammatory cytokine IL-6	Tocilizumab <sup>57</sup>
TNF- $\alpha$ monoclonal antibody	Bind and block TNF- $\alpha$ from interacting with cell surface TNF receptors.	Adalimumab <sup>118</sup> Infliximab <sup>117</sup>
Soluble TNF receptor	A soluble TNF- $\alpha$ receptor-Fc protein that prevent TNF- $\alpha$ and TNF- $\beta$ from binding to membrane bound TNF receptors	Etanercept <sup>116</sup>
Small-molecule TSHR antagonist	Binding within transmembrane region of TSHR, blocking signalling of TSH either as allosteric inverse agonist or neutral antagonist	Org 274179-0 <sup>113</sup> NCGC00229600 <sup>114</sup> NCGC00242595 <sup>60</sup>
IGF-1R monoclonal antibody	IGF-1R blocking, reduces both IGF-1R and TSHR expression	Teprotumumab <sup>115</sup>
Antioxidant	Increase reserve for selenoproteins involve in oxidation reduction activity, eg, glutathione peroxidase, thioredoxin reductase, iodothyronine deiodinases	Sodium selenite <sup>121</sup>

IGF-1R, insulin-like growth factor-1 receptor; IL, interleukin; TNF, tumour necrosis factor; TO, thyroid orbitopathy; TSHR, thyrotropin receptor.

Despite the established association of smoking with TO, the mechanism of smoking leading to TO remains less well defined. Cigarette smoke contains oxidants and radicals that cause oxidative burden systemically,<sup>107</sup> hence it has been proposed that increased production of reactive oxygen species by smoking overwhelms oxidation reduction. Smokers had significantly higher 8-OHdG levels than non-smokers in TO, suggesting smoking has a higher impact on oxidative stress in patients with TO.<sup>99</sup> Cigarette smoke extract can also stimulate HA production and adipogenesis in a dose-related manner, and the effect on adipogenesis is synergistic with IL-1.<sup>108</sup>

### ADVANCES IN THERAPEUTIC AGENTS FOR TO

The mainstay treatments for TO have been systemic corticosteroids and orbital radiation for active TO, and surgical rehabilitation for inactive TO until immunomodulators were trialled in TO targeting TSHR and IGF-1R on fibroblast, inflammatory cytokines IL-6, TNF and CD20+ B cell depletion<sup>109</sup> (table 1).

The better studied immunosuppressive therapy for TO is rituximab, an anti-CD20 monoclonal antibody that targets CD20 on B cells and its precursors. A systematic review of 43 TO cases treated with rituximab showed improvement in disease activity and severity in 91% cases, no improvement in 3 cases and worsening in 1 case.<sup>110</sup> A randomised controlled trial (RCT) in Europe comparing rituximab to intravenous methylprednisolone in active moderate–severe TO supports effectiveness and disease-modifying effects with 100% response rate, no reactivation of TO at 24 weeks and less rehabilitative surgery required at 76 weeks.<sup>111</sup> An RCT in North America comparing rituximab to placebo (ie, comparing to natural history) did not show a significant difference in the improvement of disease activity at 24 and 52 weeks, and there were more moderate-to-severe adverse events in the rituximab group.<sup>112</sup> The conflicting results from the rituximab RCTs could be related to small sample sizes and require clarification with larger RCTs.

Drug-like small-molecule TSHR antagonists are emerging as a promising new treatment for TO and GD. M22, a small-molecule TSH agonist, increased cAMP production in a TSHR-transfected ovary cell line and TO orbital fibroblasts, and the cAMP response was effectively abolished by low-molecular-weight TSHR antagonist.<sup>113</sup> The results were replicated separately where small-molecule TSHR antagonists can inhibit both basal and stimulated cAMP, pAKT and HA production in orbital fibroblast in a dose-dependent manner.<sup>60 114</sup> Teprotumumab, a humanised anti-IGF-1R monoclonal antibody, is in phase II clinical trial for moderate–severe active TO. Preliminary study shows teprotumumab can inhibit expression of TSHR and IGF-1R on CD34+ fibrocytes and TSH-induced IL6 and IL8 production by partially inhibiting phosphorylation of AKT.<sup>115</sup>

Tocilizumab, a recombinant, humanised monoclonal antibody to IL-6 receptor, has been trialled in 18 patients with active TO refractory to intravenous steroids. Tocilizumab significantly improved clinical activity score in all patients and disease activity remained stable up to 27 months after infusion.<sup>57</sup> The anti-TNF monoclonal antibodies infliximab, adalimumab and soluble TNF receptor etanercept have been trialled in small numbers of patients with active TO.<sup>116–118</sup> Etanercept seems to be effective in controlling activity of TO, leading to a marked improvement in mainly soft tissue changes reported at 60%, but up to 30% had recurrence of TO activity after treatment cessation.<sup>116</sup> Adalimumab reduced inflammatory score in 6 of 10 patients, the greatest benefit being seen in active TO with severe

inflammatory signs.<sup>118</sup> Apart from IL-6 and TNF antagonists, in vitro use of anti-IL-1 antibody has been shown to reduce adipogenesis by 82% in orbital fibroblasts exposed to cigarette smoke extract.<sup>108</sup> Novel therapeutic options for TO show some exciting developments, but large RCTs for these agents are needed to determine both efficacy and safety profile.

Antioxidants have a promising role in the treatment of mild to moderately active TO. In the first pilot study of antioxidant supplementation, allopurinol and nicotinamide therapy reduce soft tissue swelling and total eye score in 82% of patients accompanied by high patients' satisfaction in mild to moderately severe TO.<sup>119</sup> Selenium, a trace mineral incorporated into several selenoproteins and functions as antioxidant, reduces thyroperoxidase antibodies in autoimmune thyroiditis.<sup>120</sup> A subsequent double-blind, RCT of selenium supplemented for 6 months in TO was associated with improved quality of life, reduced soft tissue inflammation, improved appearance and slowed progression of TO compared with placebo.<sup>121</sup>

### CONCLUSION

Cellular immunity has an important role in orbital inflammation in TO, which involves interaction of T cells with orbital fibroblasts through specific receptor ligand bridges, with propagation of multiple intracellular signalling cascades leading to secretion of HA, adipogenesis and the release of chemotactic factors and cytokines that ensure perpetuation of orbital inflammation. TSH receptor appears the likely candidate as an autoantigen. IGF-1 receptor on orbital fibroblasts mediates some aspects of orbital changes, and importantly, it has a role in adipogenesis, HA synthesis and lymphocyte trafficking. Oxidative stress is increased in TO, and the increased oxidative burden appears to potentiate orbital inflammation, fibroblast proliferation and GAG production. With the emergence of animal models in TO and newer TSHR antibody assays, future studies will allow detailed evaluation of the heterogenous TSHR antibodies and their effects on TO, testing of new treatments targeting receptor ligand binding, signalling pathways and T and B cells. Further studies in signalling networks and molecular triggers that lead to burnout of TO will improve our understanding of TO and can in turn open future therapeutic directions.

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