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Transforming Growth Factor β (TGF-β), a master regulator of thyroid autoimmunity; what do we know and what can we hope for the future; a review;

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Transforming Growth Factor β (TGF-β), a master regulator of thyroid autoimmunity; what do we know and what can we hope for the future; a review;

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Abstract

In this article, we review the significant role of Transforming Growth Factor beta (TGF-β) in the development, evolution and treatment of thyroid autoimmunity. We first examine the pathophysiology of thyroid autoimmunity in various associated pathological conditions, then the generation and actiωation of TGF-β, the significance of its action and its important role regarding the immune system. We then examine the defects in TGF-β observed thus far in autoimmune thyroid diseases (*Grave's disease* and *Hashimotos' thyroiditis*), *thyroid-associated orbitopathy*, *autoimmune thyroid disease in pregnancy* and *post-partum thyroiditis* and the central role of TGF-β in the pathophysiology and development of the whole spectrum of thyroid autoimmunity. Finally, we point to possible clinical applications involving TGF-β as a prognostic factor and a therapeutic target for thyroid autoimmunity and future perspectives.

1. Introduction

Thyroid autoimmunity, a group of organ-specific autoimmune diseases, comprise *Graves' disease, Hashimoto's thyroiditis, thyroid-associated orbitopathy, thyroid disease in pregnancy and post-partum thyroiditis*. Research on their pathophysiology is rich focusing in particular on immune/inflammatory and endocrine mechanisms.

The superfamily of growth and transforming factors, which includes transforming growth factor beta (TGF-β), is involved in the development of thyroid autoimmunity. It seems that TGF-β, depending on its serum concentrations, the type and/or timing of expression of thyroid autoimmunity and the nature of the signalling pathway activated, plays a major role on thyroid autoimmunity, which can be either suppressive or promoting. In this review we examine the significant role of TGF-β in the development, phenotype and prognosis of the thyroid autoimmunty and point to possible future therapeutic implications of the former in the latter.

1.1. Autoimmune thyroid diseases

Graves' disease is a common autoimmune disease and the most frequent cause of persistent hyperthyroidism in adults. Genetic susceptibility as well as genetic factors are involved very often in the generation of *Graves' disease*. In the latter, autoimmune reaction induces thyrotropin receptor auto-antibodies (TRAbs) production by B-lymphocytes clones, which bound on and activate the thyrotropin receptor (THSr) on the surface of thyroid follicular cells, leading to stimulation of thyroid hormones synthesis and secretion as well as follicular cell growth and thyroid gland hyperplasia (diffuse goiter) (Figure 1)(1) Sometimes in *Graves' disease*, either initially or after a hyperthyroid period, TRAbs exert a blocking rather than a stimulating action on TSHr, inducing hypothyroidism, while the autoimmune reaction can extend to other thyroid antigens, leading to increased levels of anti-thyroid peroxydase (TPO) and/or anti-thyroglobulin (Tg) auto-antibodies. The clinical expression of *Grave's disease* comprises hyperthyroidism, goiter, thyroid-associated ophthalmopathy, orbitopathy and occasionally a dermopathy referred to as pretibial or localized myxedema (2).

Hashimoto's thyroiditis, the most common cause of hypothyroidism in iodine-sufficient areas of the world, presents with characteristic histopathological abnormalities such as profuse lymphocytic infiltration, lymphoid germinal centres, and destruction of thyroid follicles (3). Autoimmune mechanisms comprise accumulation of major histocompatibility complex (MHC) class II-positive antigen-presenting cells (APC) and of different subclasses of macrophages in the thyroid, expression of human leukocyte antigens (HLA) on thyroid cells, activation and clonal expansion of naïve T-cells, followed by a clonal expansion phase and maturation of autoreactive T- and B-cells and activation of Fas-Fas ligand interaction followed by thyroid epithelial cell apoptosis. The latter is observed in presence of increased concentrations of anti-TPO and/or

anti-Tg auto-antibodies and gradual thyroid destruction, with (goitrous autoimmune thyroiditis) or without (atrophic autoimmune thyroiditis) goiter formation (4).

T regulatory- (Treg), T helper- (Th) (more specifically Th17) and B-cells play an important role in the pathogenesis of both *Grave's disease* and *Hashimotos' thyroiditis*. This pathogenesis involves predominantly humoral and cell-mediated autoimmunity, respectively (Figure 1). Parts of the autoimmunity pathophysiology of these entitities are related, as indicated by the familial association of *Graves' disease* and *Hashimoto's thyroiditis* (5).

Thyroid-associated orbitopathy (frequently termed *Graves'* ophthalmopathy, thyroid eye disease or thyroid ophthalmopathy) is an autoimmune disorder of the extraocular muscles and surrounding orbital connective tissue, usually accompaning hyperthyroidism in *Graves' disease*, but also been observed in patients with *Hashimoto's thyroiditis* or even in euthyroid patients bearing autoimmune stigmata (6). Typical signs of *thyroid-associated orbitopathy* comprise upper eyelid retraction, periorbital oedema, proptosis, impaired eye motility, protrusion and, occasionally, optic nerve compression, restriction in extraocular motility and lagophthalmos. In case of *Graves' disease* co-expressed with *thyroid-associated orbitopathy*, TSHrs found on thyroid cells, orbital muscular cells and fibroblasts are targeted by sensitized T-cells and TRAbs. This mechanism does not explain the presence of *thyroid-associated orbitopathy* in *Hashimoto's thyroiditis* (7). Both genetic and environmental factors contribute to the pathogenesis of *thyroid-associated orbitopathy*. Autoimmune cross-reactivity against shared antigens between the thyroid cells and retro-orbital tissues initiates the immune/inflammatory cascade which is followed by infiltration of orbital muscular cells and fibroblasts by T-cells, mast cells and plasmatic cells. The autoimmune activation of orbital fibroblasts is followed by their proliferation and differentiation into myofibroblasts and adipocytes, leading to production of glycosaminoglycans (GAGs), chemokines and cytokines, which enhance the immune/inflammatory reaction resulting thus, into retro-orbital tissue enlargement (8). Activated orbital fibroblasts in *thyroid-associated orbitopathy* produce, among others, excessive amounts of TGF-β (9). Two types of not mutually exclusive thyroid-associated orbitopathy exist: type I, which is characterized by minimal inflammation and restrictive myopathy, and type II, which exhibits significant orbital inflammation and restrictive myopathy (10). Unfortunately, the prediction of thyroid-associated orbitopathy evolution is difficult and limits early treatment (11).

The commonest autoimmune thyroid diseases during pregnancy are *Grave's disease* and *Hashimoto's thyroiditis.* Thyroid physiology undergoes dramatic changes during pregnancy (due to complex adaptive changes). The latter evolve in parallel with complex physiologic changes in maternal immune system destined to develop intrauterine immune tolerance for the "non-self" fetus, while immune capacity of counteracting external pathogens is maintained. During pregnancy, B-cell activity and autoantibodies

secretion are decreased, due to the placental CRH-induced stimulation of the maternal pituitary-adrenal axis (12). Thus, during pregnancy the immune response undergoes a major shift towards increased Th2- and reduced Th1 (mainly inflammatory)- associated immunity (Th2 phenomenon) (13). This shift is partially accountable for the different phenotypes of autoimmune thyroid disease during pregnancy. Th1-mediated autoimmune diseases improve during pregnancy and worsen in post-partum. However, the Th2 predominance does not explain the decrease of autoantibodies, including thyroid autoantibodies, exemplified by the improvement of *Grave's disease* in pregnancy (14). Tregs could explain some unclear aspects of the Th1/Th2 ratio hypothesis. The pregnancy-associated changes in the maternal immune system are translated upon maternal thyroid function during this period of life (15).

Post-partum thyroiditis is a destructive autoimmune thyroiditis manifesting within the first year following parturition and considered a variant of *Hashimoto's thyroiditis*. *Post-partum thyroiditis* is usually painless and is characterized by the development of transient thyrotoxicosis and/or hypothyroidism (16). *Post-partum thyroiditis* proceeds in a sequence from a widespread thyroid cell lysis causing release of excessive amounts of thyroid hormone (toxic phase), followed by a resulting thyroid cell loss (hypothyroid phase), thyroid cell regrowth, and thyroid function recovery. The pathogenesis of *post-partum thyroiditis* involves humoral and cellular immune mechanisms, showing a rapid evolution and recovery of euthyroidism. The immunological features of *post-partum thyroiditis* comprise the presence of anti-TPO- and, less anti-Tg-auto-antibodies, abnormalities in circulating T-cell population, and goiter with lymphocytic infiltration. Women presenting with other autoimmune diseases are at increased risk of *post-partum thyroiditis*, while some women never recover from the latter and develop permanent hypothyroidism or goiter (17).

1.2. Transforming growth factor beta

1.2.1. TGF-β family and TGF-β receptors

Transforming growth factor-beta (TGF-β) subfamily consists of three isoforms (TGF-β1, TGF-β2 and TGF-β3 expressed in mammalian tissues) and belongs to the large and very important superfamily of growth and transforming factors which includes among others pleiotropic cytokines/growth factors, the activin/inhibin family, bone morphogenetic proteins, growth differentiation factors and the glial cell line-derived neurotrophic factor family (18). TGF-β isoforms contain highly conserved amino acid and are secreted by all white blood cell lineages, including the major immune/inflammatory cell lineages [dendritic cells (DCs), mast cells, macrophages, neutrophils, basophils, eosinophils, natural killer (NK) cells, T-cells, B-cells]. Transforming growth factor-beta regulates cell growth, differentiation, angiogenesis and immune system functions, such as immune tolerance, tumor rejection, and suppression of autoimmune mechanisms (19,20). All TGF-βs are synthesized as precursor molecules (biologically inactive

forms) containing a propeptide region in addition to the TGF-β homodimer. After it's synthesis, the TGF-β homodimer interacts with a Latency Associated Peptide (LAP), a protein derived from the N-terminal region of the TGF-β gene product, forming a complex called Small Latent Complex (SLC) (21). This complex remains in the cell until it is bound by another protein called Latent TGF-β-Binding Protein (LTBP), forming a larger complex called Large Latent Complex (LLC). It is this LLC that gets secreted to the extracellular matrix (ECM). In most cases, before the LLC is secreted, the TGF-β precursor is cleaved from the propeptide but remains attached to it by noncovalent bonds. After its secretion, it remains in the extracellular matrix as an inactivated complex containing both the LTBP and the LAP which need to be further processed in order to release active TGF-β. Proteases, integrins, pH, and reactive oxygen species are some of the factors that contribute to TGF-β activation (22) (Figure 2)

There are four different LTBP isoforms known, LTBP-1, LTBP-2, LTBP-3 and LTBP-4. Mutation or alteration of LAP or LTBP can result in improper TGF-β signaling. Furthermore, specific LTBP isoforms have a propensity to associate with specific LAP-TGF-β isoforms. Moreover, the structural differences within the LAP's provide different latent TGF-β complexes which are selective but to specific stimuli generated by specific activators.

The attachment of TGF-β to the LTBP is by disulfide bond which allows it to remain inactive by preventing it from binding to its receptors. Because different cellular mechanisms require distinct levels of TGF-β signaling, the inactive complex of this cytokine gives opportunity for a proper mediation of TGF-β signaling. Only following disruption of this binding, mature TGF-β is activated and can bind to its receptors (23).

Three types of TGF-β receptors (cell surface proteins which function as serine-threonine kinases) are found in most cells: TGF-β receptor I (TβRI), II (TβRII) and III (TβRIII). The latter, also called betaglycan, is the largest in molecular weight and most abundant TGF-β receptor. It is expressed on both fetal and adult tissues in most cell types. TGF-β receptors I and II mediate signal transduction, while TβRIII is not directly involved in intracellular TGF-β signalling, but it controls access of TGF-β to TβRI and TβRII, modulating thus, the intracellular TGF-β activity (20). Firstly, activated TGF-β binds to TGFβRII, which in turn binds and phosphorylates TGFβRI to initiate TGF-β signalling cascade.

Currently, some TGF-β intracellular signalling pathways are known while the full mechanism is not yet well understood. Some of the activating pathways are cell or tissue specific, while others can be found in multiple cell types and tissues (24) (Figure 3). The Smads (the homologies to the Caenorhabditis elegans SMA ("small" worm phenotype) and Drosophila MAD ("Mothers Against Decapentaplegic") family of genes) comprise a family of eight structurally similar proteins that are the main intracellular signal transducers for TβRs. Thus, the Smad (or canonical) pathway is the main signalling pathway, for TGF-β (25).

Following phosphorylation of TGF-βRI, the latter, in its turn, phosphorylates, downstream Smad2 and Smad3. Then, Smad4 is recruited and translocates to the nucleus, where it regulates the transcription of TGF-β target genes, which in case of T-cells are largely unknown (26). On the other hand, 'noncanonical or non-Smad' signalling pathway involves recruitment of Smad7 to the complex of activated TGF-βRs or phosphorylated Smad2/3C (27). The Smad canonical pathway is associated with the antiproliferative effect of TGF-β. Dysregulation of TGF-β signalling pathways contributes to several pathological processes including autoimmune disorders (28).

1.2.2. The role of TGF-β in the immune system

Every human being is born with *innate (or natural)* immunity, while *adaptive (or active)* immunity develops throughout life. In *innate* immunity, myeloid and lymphoid cells have the ability to exert rapid effector function through a limited number of germline-encoded receptors, while in *adaptive* immunity, T- and B- cells clonally express a large variety of antigen receptors, produced by site-specific somatic recombination (29).

TGF-β regulates homeostasis and affects cellular components of *innate* [such as white blood cells (DCs, macrophages, neutrophils, basophils, eosinophils, mast cells and natural killer (NK) cells)] immunity (Table 1). Dendritic cells have a complex and mutual relationship with TGF-β. They play a crucial role in the activation of TGF-β, *via* multiple factors (30). Of interest, TGF-β overexpression supresses maturation and function of DCs and hampers their migration to other tissues (31). Macrophages, myeloid immune cells, can easily suffer a switch in their function depending on their external microenvironment. Thus, they are called M1 (classical or pro-inflammatory) or M2 (alternative macrophages) (32). TGF-β inhibits the proinflammatory response of macrophages as well as their cytokine-induced cytokine stimulation and induces their migration, enhancing their adherent properties. The regulation of macrophages by TGF-β is directly influenced by the macrophage identity. The activation of TGF-β-secreting M2 macrophages is promoted by TGF-β itself. On the other hand M2 macrophages are essential for TGF-β-driven inflammatory functions (33). NK-cells are cytotoxic lymphocytes which interact with naïve T-cells and regulate their responses *via* TGF-β production (34). TGF-β inhibits NK-cell proliferation and function, an action partly modulated by Tregs, known to produce increased amounts of TGF-β. Blockade of TGF-β signaling in NK-cells causes NK-cells accumulation (35). TGF-β induces chemotaxis of both neutrophils (36) and eosinophils (37). In addition, TGF-β can induce chemotaxis and enhance the adherent properties of mast cells, while it can promote or suppress the latter function (38).

TGF-β regulates homeostasis and affects, also, cellular components of *adaptive* (T- and B-cells) *immunity* (39). T-cells belong to two categories, *conventional adaptive* and *innate-like* T-cells. The former are subdivided into subsets according to their functional role (40) (Table 2): i) T-helper cells (Th cells or

$CD4^+$), further subdivided into Th1, Th2, Th9, Th17, Th22 and Tfh cells, according to the type of cytokine they produce and their role in immune defense and autoimmunity ; ii) cytotoxic $CD8^+$ T-cells (CTLs); iii) memory T-cells, which can be either $CD4^+$ or $CD8^+$ T-cells and comprise central memory T-cells (TCM cells), effector memory T-cells (TEM cells and TEMRA cells), tissue resident memory T-cells (TRM cells), and virtual memory T-cells; and iv) T regulatory cells (Tregs or suppressor T-cells classified into natural Tregs (nTregs or $CD4^+CD25^+$ T-cells) and adaptive (induced) Tregs (iTregs). TGF-β exhibits immune-promoting and -suppressing properties regarding *adaptive* T-cell immunity (41). The former participates in the activation of naive T-cells. Following stimulation by TGF-β, naive $CD4^+$ T-cells become effector T-cells (a group of T-cells including several types of the latter such as $CD4^+$, $CD8^+$, Th- and Treg cells, which actively respond to a stimulus) (42). Subsequently, TGF-β suppresses proliferation and differentiation of effector T-cells *via* inhibition of Th2-produced IL-2 (43). Interestingly, TGF-β by altering the type of cytokine produced, mediates phenotypic metamorphosis among effector T-cells (44). Furthermore TGF-β enhances proliferation of $CD8^+$ cells under certain conditions in experimental mouse models and increases TNF production by both $CD4^+$ and $CD8^+$ cells (45). Moreover, TGF-β is critical for the transformation of nTregs to iTregs *via* increased Foxp3 (Forkhead-Box-Protein P3), also Scurfin) expression in the former (46). Interestingly TGF-β can also promote the Treg-induced inhibition of the exocytosis of granules and inhibit the generation and activation of CTLs and supress the cytotoxicity of the latter *via* transcriptional regression of genes encoding proteins, which are vital for CTLs' function (47). Conclusively TGF-β exerts a pleiotropic function on the generation, proliferation, activation, function and apoptosis of T-cells depending on their as well as their extra- and intra- cellular microenvironment (cytokinic and cellular milieu) (Figure 4).

B-cells represent the second major category of adaptive immunity cells. These lymphocytes develop in several stages from hematopoietic stem cells, that originate from bone marrow. B-cell activation, which can be T-cell-dependent and -independent occurs in the secondary lymphoid organs, such as the spleen and lymph nodes (48). These cells function in the humoral immunity component of the adaptive immune system by secreting antibodies. B-cells express high-affinity TGF-β receptors while they secrete TGF-β (49). This growth factor inhibits B-cell activation and antibodies production, while it promotes class switching of IgA in both human and mouse B-cells and inhibits immunoglobulin synthesis and class switching to the majority of IgG isotypes. TGF-β induces apoptosis of immature or resting B-cells by an unknown as yet mechanism, which may overlap with its anti-proliferation pathway (50).

Collectively, TGF-β exerts multi-faceted effects on various immune functions. The exact stimuli that trigger TGF-β secretion and modulate its action into such a broad spectrum of functions must be further

studied and might prove the 'key' to unlock the 'hidden' pathways of immunity and reverse the detrimental effects of autoimmunity .

2. Materials and Methods

To identify publications regarding the role of TGF-β in autoimmune thyroid diseases, a systematic literature search for human studies was conducted in five electronic databases (PubMed, Cochrane, Medline, Scopus and EMBASE) until August 2020 and using combinations of the key terms "TGF-β" and "autoimmune thyroid diseases" . On the top, a manual search of key journals and abstracts from the major annual meetings in the fields of thyroidiology, immunology and endocrinology was conducted.To date, the number of published research data, regarding the effect of TGF-β on thyroid autoimmunity is limited and in some cases controversial.

3. TGF-β and autoimmune thyroid diseases

Thyroid gland homeostasis and function is maintained through a fine regulation of thyrocyte growth and differentiation, which is achieved *via* a complex network of factors that act through endocrine, paracrine, or autocrine mechanisms and depends on interactions between TSH and various growth factors, among them those belonging to TGF-family and particularly TGF-β (51). The proliferation and differentiation of thyroid epithelial cells are under the control of a positive systemic signal, TSH, and a negative locally produced signal, TGF-β. Intact follicular thyroid cells produce normaly TGF-β, which acts locally in a paracrine and/or autocrine fashion (52). Thyrocytes produce different proteases, which can activate TGF-β. In animal models with TSH-induced thyroid hyperplasia, the expression of TGF-β is upregulated, leading to temporal stabilization of goiter size (53). TβRI and II are both expressed on thyrocytes allowing the the activation of the TGF-β/SMAD pathway and the subsequent TGF-β transcription (54) Furthermore TGF-β is an important negative regulator of thyrocytes, antagonising the mitogenic effects of the main growth factors in human, dog, pig and rat thyroid cultured cells. TGF-β delays progression of the thyrocytes proliferation and down-regulates the expression of thyroid-specific genes in most of the species. TGF-β inhibits iodide uptake and metabolism, cAMP formation, and thyroxin release from the thyroid cells in human and animal cultures (55). In addition TGF-β inhibitis TG biosynthesis and TSHr expression and counteracts TSH positive effect on folliculogenesis in the thyoid gland (56). Finally TGF-β inhibits MHC class I in thyrocytes and indirecty supresses TSHr and MHC class I and class II expression (57) Therefore, it can be easily supported that TGF-β exerts a malor regulatory effect on thyroid cells, mainly inhibiting their proliferation and limiting function. Of note TGF-β expression and activity regarding thyroid function are clearly regulated by sex hormones. Estrogen effects on follicular thyroid

cells are mediated by TGF-β synthesis and secretion by thyroid stromal cells, with the latter expressing both types of estrogen receptors (ER-a and ER-b) (58). In detail estrogens enhance both synthesis and secretion of TGF-β by thyroid stromal cells, inducing TGF-β activation, *via* Smad2 phosphorylation, while on the other hand TGF-β inhibits estrogen receptors (ER) expression *via* Smad4, which co-represses ER expression (59). ER-β activation mediates TGF-β mediated Th-17 type responses (60) while on the contrary ER-α blocks TGF-β signaling *via* depletion of the Smad 2/3 protein concentrations (61). Furthermore androgens suppress TGF-β transcriptional responses by impeding the binding of Smad3 to the SBE (Smad binding element) (62). In addition progesterone antagonizes TGF-β induced Smad activation and inhibits TGF-β/SMAD regulated genes expression (63). Dysruption of the sex hormones mediated TGF-β regulation is directly implicated in the pathophysiology of thyroid autoimmunity, especially in women. Genetic background and disturbances of the immune regulatory mechanisms contribute to the development of autoimmune thyroid diseases. TGF-β has been implicated in these mechanisms**.** Data indicate that the severity of *Hashimoto's disease*, the intractability of *Graves' disease*, the gravity of *thyroid-assocciated orbitopathy* and that of autoimmune *thyroid diseases during pregnancy* and the course of *post-partum thyroiditis* vary among patients reflecting the significant pathophysiologic role of TGF-β as master regulator of autoimmune thyroid diseases (Table 3)**.**

3.1. TGF-β and Graves' disease

Normally, TGF-β inhibits iodide uptake, thyroid hormone biosynthesis and secretion, thyroglobulin and thyroid peroxidase expression, proliferation of thyroid cells (even in species such as rats, pigs, dogs, and humans), and TSH receptor expression, while it modulates T- and B- cell activation and differentiation (51). Thus, its deficiency contributes to the pathogenesis of *Graves' disease*. Indeed, polymorphisms at codons 10 and 25 of the TGF-β gene represent risk factors for the development of *Graves' disease.* Significant relationships between TGF-β genotypes and clinical/laboratory findings in *Graves' disease* have been described (64). *Graves' disease* is characterized by increased proliferation of thyroid follicular cells, increased TRAbs against TSH receptor and thyroid lymphocytic infiltration. TGF-β affects the interaction between thyroid follicular cells and T lymphocytes in these patients by exerting potent immunosuppressive effects *via* inhibition of pro-inflammatory cytokines, such as TNF and IL-1, secreted by immune cells and by blocking cytokines-induced adhesion molecules (i.e. intercellular adhesion molecule 1, ICAM1; vascular cell adhesion molecule 1, VCAM1) (65). Furthermore, TGF-β coud be also implicated in *Graves' disease* etiopathogenesis by decreasing the IFNγ-induced expression of human leucocyte class II antigens (HLA). This growth factor inhibits the proliferation and differentiation of T-cells, alters both Th1 cell and Th2 cell-mediated responses, suppresses B-cells and decreases antibody production but also induces and sustains normal physiologic suppression activity of Tregs, regulating thus, T-cell activation and playing a major role

in peripheral tolerance to self-antigens (66). Depletion of Tregs in mice increases susceptibility to experimental *Graves' disease*; thus, deficiency (or decreased expression) TGF-β could contribute to the development of *Graves' disease*. When administered to patients with Graves' disease, human recombinant TGF-β (hrTGF-β) inhibits proliferation of peripheral blood mononuclear cells as well as of peripheral and thyroid-derived T-cell clones, while it suppresses the recognition of thyroid follicular cells by thyroid autoantigen specific T-cell clones (67). Inhibition of autoantigen recognition was observed when hrTGF-β was added to thyroid follicular cell/lymphocyte co-cultures or when thyroid follicular cell cultures were pre-incubated with hrTGF-β, probably a result of decreased antigenicity of target T-cells. Of note, addition of hrTGF-β suppressed thyroid peroxidase and HLA class II autoantigen expression in cultures of thyroid follicular cells (67). However, in certain studies no significant differences were found in plasma TGF-β concentrations between patients with *Graves' disease* and healthy controls (68).

Interestingly, the +869T/C polymorphism in the TGF-β gene, in the region encoding the signal peptide, results to increased circulating concentrations of TGF-β and has been associated with the intractability of *Graves' disease* rather than the susceptibility to it (69). Patients with intractable *Graves' disease* present more often with the two-allele genotype of this polymorphism than patients in remission.

Further deep research is to be conducted in order to trully understand the amount of interference of TGF-β in the generation of *Graves' disease*.

3.2. TGF-β and Hashimoto's thyroiditis

In *Hashimoto's thyroiditis* pathophysiology, immunologic events, such as interactions between thyroid-specific autoantibodies and Th cells (predominantly Th1, Th17 cells and iTregs), are clearly regulated by TGF-β (70). At the initial pathophysiologic stage of *Hashimoto's thyroiditis*, thyroid gland is infiltrated by APCs which attack thyrocytes. The latter release thyroid-specific proteins, which are subsequently attached on the cell surface of APCs. Only then, APCs can travel from the thyroid to the draining lymph node. At this stage, decreased TGF-β concentrations were documented (71). Deficiency or decreased expression of TGF-β contributes to the development of *Hashimoto's thyroiditis via* alteration of Th1 cell and Th2 cell-mediated responses and attenuation of normal physiologic suppression activity of Tregs. More specifically, increased concentrations of TGF-β lead to higher and faster proliferation of autoimmunity-suppressor Th10 cells (72). TGF-β-induced T-cells can educate naive $CD4^+$T-cells into development of suppressive properties by generating Tregs. The latter are critical for maintaining peripheral tolerance and secrete in their turn, different antimitogenic ligands such as TGF-β itself. Activation of TGF-β-producing iTregs prevents the development of experimental autoimmune thyroiditis. Transgenic enhancement of a selective cytotoxic T-lymphocyte antigen 4 (CTL-4) in an experimental rodent model of *Hashimoto's thyroiditis*, induced increase of antigen-specific

$CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^-TGF\text{-}\beta^+$ adaptive Tregs, followed by increased production of TGF-β and finally, led to suppression of the disease (73). The decreased serum TGF-β concentrations at this stage of the disease, are not altered by levothyroxine replacement in these patients (72).

TGF-β exerts a different role on pathogenesis of *Hashimoto's thyroiditis*, based on the pathophysiologic stage of this disease. In detail, at the initial pathophysiologic stage of *Hashimoto's thyroiditis*, APC, autoreactive (AR) T-cells and B- cells interact, resulting to thyroid autoantibodies production by B-cells. At this stage, thyroid gland is infiltrated from antigen-producing B-cells, cytotoxic T-cells and macrophages, which accumulate in the thyroid through expansion of lymphocyte clones and propagation of lymphoid tissue within the thyroid gland, a process mediated by Th1 cells. At the final pathophysiologic stage of *Hashimoto's thyroiditis*, autoreactive T-cells, B-cells and antibodies cause massive depletion and apotosis of thyrocytes leading to hypothyroidism (74,75). Of note, while at the initial pathophysiologic stage of *Hashimoto's thyroiditis* TGF-β plays an autoimmunity-suppressor role, at the final stage of the disease it may trigger fibrosis as shown in an experimental granulomatous thyroiditis model (76). Thereby, at this stage of the disease, abnormaly elevated TGF-β serum concentrations are associated with increased fibrotic activity. Although TGF-β is generally considered as the key growth factor favoring normal tissue remodelling after injury, the "dark-side" of this property is the development of tissue fibrosis as a result of excessive TGF-β production. Indeed, the use of anti-TGF-β antibody in an experimental granulomatous thyroiditis model resulted in attenuation of fibrosis (76). Of note also that treatment of stromal thyroid cells with estrogens and more specifically with 17-estradiol and subsequent ER-β activation stimulates the development of experimental autoimmune thyroiditis in mice (59). On the contrary treatment with estrogen antagonists as coumestrol suppress the production of thyroid-specific autoantibodies in the development of experimental autoimmune thyroiditis, thereby limiting the gravity of thyroid autoimmunity (60) The significantly different concentrations of TGF-β between atrophic and non-atrophic variants of *Hashimoto's thyroiditis* might explain histopathological differences in the stages of thyroiditis (77).

Genetic polymorphisms of TGF-β cause reduced tolerance against several self-antigens in case of thyroid-associated orbitopathy in patients with Hashimoto's thyroiditis. Two SNPs in TGF-β gene exon 1 (positions +869 and +915) change the amino acid sequence of the signal peptide, affecting thus, the level of expression and secretion of TGF-β. Transition at codon 25 of GNC (ArgNPro substitution) reduces the secretion and plasma concentrations of TGF-β and results in a 2-3-fold increased risk for *Hashimoto's thyroiditis*. The interaction of this growth factor gene polymorphism at codon 25 with other genetic and environmental factors may be related with increased susceptibility to *Hashimoto's thyroiditis* (78). The significance of the LeuNPro substitution at codon 10 is controversial, most

probably reducing also TGF-β concentrations. Furthermore the +869T/C polymorphism in the TGF-β gene is associated with the severity of the disease and with variable TGF-β secretion, depending on the respective allele (T or C) (79).

Conclusively the vital effects of TGF-β on *Hashimoto's thyroiditis* development, vastly different based on the pathophysiologic stage of the latter, need to be further researched in depth, in order to provide a viable therapeutic strategy for the treatment of *Hashimoto's thyroiditis*.

3.3. TGF-β and thyroid-associated orbitopathy

TGF-β is an important regulator of the immunological and inflammatory pathways leading to the the development of *thyroid-associated orbitopaty*. This growth factor, in one hand, contributes to tolerance against self-antigens, while on the other hand, it induces orbital myofibroblast proliferation and differentiation *via* HAS1 and HAS2 expression (80). Perimysial orbital fibroblasts uniformly express CD90, a cell surface glycoprotrin, whereas orbital fibroblasts in adipose tissue can be distinguished in of both CD90-positive and CD90-negative cells. The former differentiate into myofibroblasts following TGF-β stimulation (81). In *thyroid-associated orbitopathy*, TGF-β induces proliferation of myofibroblast and cytokine production as well as hyaluronan production while it contributes to TSHr degradation and to adipogenesis decelaration (82). In addition, TGF-β increases expression of sphingosine-1-phosphate (S1P), a pro-fibrotic effector for *thyroid-associated orbitopathy* fibroblasts, thus contributing to ocular fibrosis (83). Furthermore, TGF-β stimulation contributes to the matrix expansion, a characteristic of active *thyroid-associated orbitopathy*. In the latter, a positive correlation exists between hyaluronic acid and TGF-β in orbital fibroblasts (84). It was recently observed that a fibroblast subpopulation, which produces prostaglandin E2, IL-8 and HA, when exposed to TGF-β (which is strongly expressed in the orbit of patients with mild and severe *thyroid-associated orbitopathy*), differentiates into myofibroblasts, participating in repair and fibrosis (85) (Figure 5). Expression profiling of TGF-β-treated orbital tissue and ocular fibroblasts derived from *thyroid-associated orbitopathy* patients, revealed up-regulation of genes potentially relevant to this disease, including that encoding serine protease inhibitor PAI-1. The latter is a downstream effector of the fibrotic response, which limits matrix degradation, promotes tissue fibrosis and duration and amplitude of the inflammatory response (80). TGF-β synthesis is stimulated by exogenously delivered PAI-1 in several cell types, suggesting the existence of a PAI-1/TGF-β-positive feedback mechanism and a potential pro-fibrogenic "loop" (86). In addition, specific TGF-β polymorphisms (codon 10, +869C e/ T codon 25, +915G/C) increase by 2-3 fold the risk for *thyroid-associated orbitopathy* development, *via* altered expression of TGF-β followed by inhibition of the suppressive function of Tregs on autoimmunity (87). Further research regarding the vital role of TGF-β regarding the development and evolution of *thyroid-associated orbitopathy* is needed.

3.4. TGF-β and autoimmune thyroid diseases in pregnancy and post-partum

3.4.1. TGF-β in autoimmune thyroid diseases during pregnancy

Transforming growth factors and especially TGF-β have been located at the human fetomaternal interface (tropho- and syncytiotropho- blast) where they contribute to the proliferation and differentiation of trophoblasts. There, TGF-β exhibits autocrine, paracrine, and endocrine functions (88). Both the placenta and the uterus produce TGF-β, while the former demonstrates high tissue affinity for maternal TGF-β (89). The latter, is largely produced by uterine Natural Killer (uNK) ($CD56^{++}/CD16^{-}$) cells, plays a key role in angiogenesis and immunoregulation during pregnancy, contributes to maternal immunosupression *via* induction of the M2-phenotype of placental macrophages, and increases placental expression of FOXP3. The latter directs naïve $CD4^{+}$ T-cells towards a Treg phenotype (90). Tregs proliferate peripherally after encountering foreign antigens (such as fetal antigens), migrate towards the feto-maternal interface and generate elevated TGF-β concentrations, resulting to immune suppression and immune tolerance of the fetus. Maternal plasma TGF-β concentrations are greater during pregnancy than in non-pregnant state as observed in murine models but there is no consensus on its quantitative evolution during pregnancy (91). It seems that, during pregnancy, maternal circulating concentrations of TGF-β are greater in the first and second compared to the third trimester. Indeed, recently, we reported that TGF-β concentrations are smaller in the 36th gestational week compared to those at the 24th gestational and the 1st post-partum weeks (92). Interestingly, in our study, mean concentrations of TGF-β at the observation time-points evolved in a “mirror” image fashion regarding the corresponding mean cortisol concentrations, suggesting a possible physiologic interplay between TGF-β and glucocorticoids during pregnancy. Of note, polymorphisms of TGF-β, such as C-509T, which increase TGF-β transcription ‘protect’ pregnant women from the development of autoimmune thyroid disease (93).

Furthermore it was observed, that serum concentrations of TGF-β (total and active form) fluctuate significantly during pregnancy in antithyroid-antibodies (ATA) positive (ATA+) and negative (ATA-) pregnant women (94). Causes of this phenomen comprise a reduced B-cell reactivity, at least in part due to placental steroids, a major shift in T-cell control expressed via decreased T1/T2 and interferon-/IL-4 ratio and increased apoptosis of lymphocytes, as mentioned above. The former could explain the observation, that serum total TGF-β concentrations are similarly distributed in ATA^{+} and ATA^{-} women, indicating that immune T-cells exert their control role on TGF-β production during pregnancy. On the other hand almost half of the ATA^{+} pregnant women but few of the ATA^{-} pregnant women present with detectable active TGF-β concentrations, highlighiting the major role of autoimmunity, mirrored in the ATA concentrations, to the activation of TGF-β. Interestingly, women with a TGF-β gene mutation (most notably of the C-allele)

present with greater ATA concentrations than pregnant women with the wild-type of TGF-β gene, even when the concentrations of this growth factor are undetectable. Supposingly conditions triggering autoantibody production are associated with TGF-β activation (95). This growth factor is increased during the toxic phase of *autoimmune thyroid diseases during pregnancy*, suggesting that the immune/inflammatory phenomena could be responsible for both TGF-β activation and the increase in autoantibody titer (96). Conclusively the role of TGF-β in case of *thyroid disease during pregnancy* is important, poorly researched and needs to be further clarified.

3.4.2. TGF-β and post-partum thyroiditis

Post-partum thyroiditis is a transient form of thyroiditis. It is characterized by thyroid cell destruction caused by specific thyroid T-cells and followed by their clonal suicide (*via* programmed cell death) (97). By its involvement in Treg homeostasis, TGF-β leads to confinement of *post-partum thyroiditis* (98). Interestingly, recently, we reported that the difference between the decreased concetration of TGF-β at the 36th gestational week and its increased concentration at the 1st week post-partum is the best negative predictor of post-partum anti-TPO antibodies concentration indicating a direct and/or indirect immunosuppressive role of TGF-β upon post-partum elevation of anti-TPO antibodies (92). Active TGF-β increases during the toxic phase of *post-partum thyroiditis*, indicating involvement of inflammation in its activation (94). *Post-partum thyroiditis* evolves through an initial transitory hyperthyroid phase followed by a hypothyroid phase, demonstrating in the end thyroid cell regrowth and thyroid function recovery. How the immune system is able to regain its equilibrium and allow the thyroid gland to recover from post-partum thyroid disease remains unclear. Possible mechanisms explaining the transience of *post-partum thyroiditis* and the ability of the immune system to regain its equilibrium involve the clonal suicide (*via* programmed cell death) of thyroid-specific T-cells following thyroid cell destruction and the induction of antigen-specific Tregs (99). Connecting point of these mechanisms is hypothesized to be the active involvement of apoptotic cells in the suppression of the inflammatory response *via* induction/release of TGF-β, among others (100). Serum TGF-β concentration is reduced between the first transitory hyperthyroid phase and the following hypothyroid phase of post-partum thyroiditis. During inflammation in post-partum thyroiditis, TGF-β is produced intra-thyroidally and activated subsequently as described before. Thus, increased circulating concentrations of TGF-β are observed following the thyroid cell destruction in the hyperthyroid phase of *post-partum thyroiditis*. During this phase, the activation of TGF-β is enhanced, while part of the circulating TGF-β may result from the damage and repair of thyroid cells, which triggers TGF-β production (94).

The evolution of *post-partum thyroiditis* seems to be closely related to TGF-β concentrations. Thus, certain authors have proposed this growth factor, as a potential predictive marker of the evolution

towards healing of *post-partum thyroiditis* (101). At post-partum the concentrations of serum total TGF-β are similarly distributed in ATA^+ and ATA^- women, indicating that the production and activation of TGF-β does not depend on B-cell physiology at this specific period in contrast to the non-pregnant state, in which B-cells regulate partly TGF-β concnentrations (102). In conclusion, increased serum concentrations of this growth factor may contribute to a complex endogenous, but still unknown, anti-inflammatory mechanism which ultimately might protect the thyroid gland from permanent immunological damage during the post-partum year (103). Further studies of serum TGF-β concentrations and their fluctuation in women with persistent hypothyroidism after the post-partum year are needed to verify this hypothesis.

4. Discussion-Eye to the future

Autoimmune thyroid disease is the most common organ specific autoimmune disorder usually resulting in dysfunction (hyperfunction, hypofunction or both) of the thyroid gland. While the immunological mechanisms involved in *Graves' disease* and *Hashimoto's thyroiditis* are closely related, their phenotypes vary due to different immunological responses. *Thyroid-associated orbitopathy* is a common extrathyroidal autoimmune manifestation of *Grave's disease* but can also occur in hypo- or eu-thyrodism with mild to severe effects on the orbit and extraocular muscles. In pregnancy, in one hand, normal thyroid physiology is altered finding a new homeostasis, while, on the other hand, autoimmune thyroid diseases manifest. The latter contribute to negative outcomes such as recurrent miscarriage (93). *Post-partum thyroiditis*, a destructive form of thyroiditis, usually presenting in the first year pospartal, is often transient, nontheless associated with a considerable risk of permanent hypothyroidism.

Transforming Growth Factor b plays a crucial and yet not fully understood role in thyroid autoimmunity. Furthermore, TGF-β suppresses specific autoimmune pathways in persons predisposed to develop thyroid autoimmunity and leads to remission. Thus, changes of its concentrations correlate significantly with the occurrence of autoimmune thyroid disease. Depending on the type of autoimmunity involved, TGF-β exerts a different but significant role regarding the development of the disease (Table 4). Thus, TGF-β deficiency contributes significantly to *Graves' disease* etiopathogenesis (64) and development while TGF-β genetic polymorphisms are associated with the intractability of *Graves' disease* (69). Furthermore, decreased TGF-β concentrations are associated with 2-3-fold increased risk for Hashimoto's thyroiditis (71). In addition, certain TGF-β genetic polymorphisms lead to increased susceptibility to and severity of *Hashimoto's thyroiditis* (78,79). It is noteworthy, that, although TGF-β acts predominantly as an immune suppressive factor in most circumstances, it can also contribute to the expansion and maintenance of specific immune cell functions. The contribution of TGF-β concentrations as a prognostic marker of evolution can be also suggested in *Graves' disease*. The TGF-β concentrations are significantly

higher in elderly patients with goiter and hyperthyroidism in comparison with healthy age-adjusted subjects. Thus, its concentrations could be useful in early diagnosısc of patients with atypical thyroid enlargement and subclinical hyperthyroidism (85). In any case, TGF-β concentrations alone or concomitantly with other factors, such as antithyroid antibodies, could be employed as marker for the diagnosis and/or the prediction for the evolution with or without treatment of autoimmune thyroid disease.

In case of *thyroid-associated orbitopathy*, TGF-β exerts its disease promoting activity *via* enhanced proliferation of myofibroblast and cytokine production as well as increased hyaluronan production resulting to exacerbated fibrosis. Specific TGF-β genetic polymorphisms increase the risk for *thyroid-associated orbitopathy* development (87). Because the extended half-life and the concentrations of TGF-β contribute to the development of fibrosis in *thyroid-associated orbitopathy*, its measurement could be used in the prognosis and treatment of this pathologic entity.

During normal pregnancy, maternal TGF-β concentrations increase, being greater in the first and second than the third trimester, eventually contributing to immune tolerance (92). Genetic TGF-β polymorphisms enhance its immunoprotective role regarding a*utoimmune thyroid disease during pregnancy.* On the other hand, other polymorphisms result into increased autoantibody production and exacerbated autoimmune response (93). In *post-partum thyroiditis*, TGF-β exerts direct and/or indirect immunosuppressive role upon post-partum elevation of anti-TPO auto-antibodies, while TGF-β activation is observed during the hyperthyroid phase of *post-partum thyroiditis (94)*. Furthermore, TGF-β concentrations could be a predictive marker of *post-partum thyroiditis* evolution towards healing. Furthermore, increased TGF-β concentrations in late pregnancy might indicate increased susceptibility to *post-partum thyroiditis* development and risk for permanent autoimmune thyroid disease in the post-partum years (101).

4.1. Therapeutic effects of TGF-β on thyroid autoimmunity

Concentrations of TGF-β alone or along with other markers, such as ATA, could be employed for the diagnosis, therapeutic follow-up and prognosis of certain autoimmune thyroid diseases. For example, TGF-β concentrations were significantly greater in elderly patients with atypical thyroid enlargement and subclinical hyperthyroidism subsequently evolving to patent *Graves' disease*, than in control age-adjusted subjects with the same initial clinical presentation, who did not develop the disease (83). Thus, TGF-β concentrations could be useful as a marker for early diagnosis and consequent therapy initiation in patients with atypical symptoms of hyperthyroidism at risk for development of *Graves' disease* (84). Also, in *thyroid-associated orbitopathy*, measurement of TGF-β concentrations could be used in the prognosis of fibrosis as well as the follow-up of the treatment of this pathologic entity (87). Furthermore, increased

TGF-β concentrations during the third trimester of pregnancy might indicate greater risk for development of *post-partum thyroiditis* (92). Unfortunately, serum TGF-β concentrations have not been proven a reliable marker of *Hashimoto's thyroiditis'* evolution (72).The involvement of TGF-β in the pathophysiology of thyroid autoimmunity made it a common research subject regarding therapeutic interventions . More specifically, various stages in the synthesis, activation and final action of TGF-β could be possible targets for therapeutic intervention. As a result, therapeutic strategies against TGF-β could be applied in ligand level (TGF-β activation), ligand–receptor level (TGF-β transcription) and intracellular level (TGF-β action) ((Table 5).

In one hand, the suppressive/ inhibitory role of TGF-β regarding immune and neoplastic pathways is exploited to achieve remission of specific diseases. Specifically:

- In multiple sclerosis, when iTregs, generated in healthy subjects, were transferred to patients with active disease, they were shown to effectively suppress autoimmune reaction via increased TGF-β production (104).

- In atherosclerosis, it has been shown that TGF-β inhibits both migration and proliferation of smooth muscle cells macrophages, protects endothelial function, reduces adhesiveness of the endothelium for inflammatory cells and contributes to vascular protection. Low concentrations of this molecule are associated with increased cardiovascular mortality. Thus the use of TGF-β is proposed for prevention of ischemic heart disease and minimization of cardiovascular death risk (105).

- In normal prostatic epithelial cells, TGF-β induced apoptosis and transcriptional responses are inhibited by androgens, thus promoting their viability. Androgens repress selectively binding of Smad 3 to SBE, thus blocking this TGF-β activation pathway. Indeed, in cultured human prostate cancer epithelial cells, the addition of TGF-β has been shown to counteract the tumor-promoting effects of ligand-bound androgen receptors and limit carcinogenesis (62).

B. **On the other hand, the enhancing/promoting role of TGF-β, in certain stages of diseases can be therapeutically targeted**. Novel therapies developed specifically to suppress/inhibit TGF-β signaling pathways can limit TGF-β biosynthesis, activation, ligand- and receptor- binding and counteract effectively its downstream responses and activity. In detail:

- In cancer therapy (malignant glioblastoma, prostatic cancer, melanoma e.t.c.), TGF-β blockade, suppresses metastasis via targeting tumour-initiating cells, enhancing immune surveillance and cytotoxic T cell activity, modulating the tumour stroma and suppressing angiogenesis. In clinical practice, therapies involving antisense oligonucleotides (ASOs), ligand traps, antibodies, small-molecule chemical inhibitors, antisense genes, pyrrole-imidazole polyamide DNA binders have been already used or are tested in clinical trials. (65).

- In fibrosis, TGF-β signalling inhibition via gene transfer of the inhibitory Smad7 has already been used in conditions such as vascular remodelling, diabetic kidney disease, and colonic and hepatic fibrosis (107).

- In various conditions such as glomerular proteinuric nephropathy, sarcoidosis, peripheral neuropathy, sickle cell disease, alcoholic steatohepatitis and radiation-induced fibrosis, downregulation of TGF-β expression leads to equilibration of Th1/Th2 imbalance, and attenuation of Th1-mediated immune reactions. This downregulation of TGF-β has been therapeutically achieved via pentoxifylline (1-5-oxohexyl-3,7-dimethylxanthine or PTX), a methylxanthine derivative (108).

- In bronchopulmonar dysplasia in preterm infants, TGF-β-induced Smad2/3 activation and genes expression has been inhibited in a dose-dependent manner via therapeutic use of progesterone (63).

-In an experimental model of hepatectomized mice for liver regeneration, TGF-β-mediated fibrosis is avoided via small peptides, which bind to a conserved sequence in the LAP region of the TGF-β latent complex, inhibiting thus, TGF-β activation and signalling (109). This method could be tested in thyroid autoimmune diseases which involve development of fibrosis (either within the thyroid or retro-orbitally).

Thus, modification of TGF-β cell physiology could be an interesting therapeutic target regarding thyroid autoimmunity. However, as yet, no therapy based on the involvement of TGF-β in thyroid autoimmune diseases is officially approved by FDA or EMAS. The dual aspect of involvement of TGF-β in thyroid autoimmunity (immune -suppressive or -promoting) should be taken into consideration. In detail:

Regarding the immune-suppressive role of TGF-β:

- addition of hr-TGF-β to human cultures of follicular thyroid/lymphocyte cells from patients with *Graves' disease* leads to thyroid autoimmunity suppression via decreased antigenicity of target T-cells and suppressed TPO and MHC class II auto-antigen expression (65)

- addition of TGF-β in cultures of thyroid cells markedly increases FOXP3 expression and leads to vigorous decrease of IL-2 expression, leading to suppression of thyroid autoimmunity (110). Thus, stimulation of polyclonal human $CD4^+$ cells with TGF-β induces human $CD4^+CD25^-$ cells to $Foxp3^+CD4^+CD25^+$ suppressor T-cells. Interestingly, following repeated stimulation with TGF-β, some Tregs express membrane-associated TGF-β which leads to strong suppression of T-cell proliferation. In this way, TGF-β could exert a prolonged and cumulatitive suppressive effect upon thyroid autoimmunity and it could be used therapeutically.

- treatment with low-level laser therapy (LLLT) is known to suppress autoimmune diseases via regeneration of various tissues. In patients with *Hashimoto's thyroiditis*, therapeutical application of LLLT (10 sessions on the skin corresponding to the thyroid gland area, twice per week) stimulates TGF-β gene

expression. This procedure could potentially limit the autoimmune process in the initial stages of *Hashimoto's thyroiditis* via significant increase of serum TGF-β concentrations (11).

Regarding the immune-promoting role of TGF-β:

- thyroid fibrosis in animal models of EAT is limited via suppression of the Smad-induced transcription of TGF-β gene. This is achieved via triiodothyronine (T3) administration and binding of the T3-thyroid receptor complex on the thyroid hormone response element (112). Similar results could be achieved by pharmacologically designed nuclear thyroid receptor ligands deprived of thyroid hormone activity (113).

- monoclonal anti-TGF-β antibodies administered to animal models of *Hashimoto's thyroiditis* suppress the final pro-fibrotic pathophysiologic stage of the disease via neutralization of the excess extracellular TGF-β (114).

- treatment of stromal thyroid cells in mice with 17-estradiol (E2)-induced ER-β activation stimulates development of EAT in mice. Treatment with coumestrol, an E 2 antagonist, limits production of thyroid-specific autoantibodies in EAT (60) while it inhibits TGF-β-mediated Th17-type response (enhances "cell-type" autoimmunity) (59). Of note, ER-α activation can oppose the effects of TGF-β activity via a novel non-genomic E2 -dependent mechanism (61).

-TGF-β-enhanced inflammatory process leads to increased expression of cyclooxygenase (COX-2) in *Hashimoto's thyroiditis*. Thus, COX-2 inhibition via Celecoxib, a COX-2 inhibitor, might be proposed as therapeutic mechanism for suppression of autoimmunity in *Hashimoto's thyroiditis* (115).

- furthermore, in cultures of human myoblasts from extraocular muscles from patients with *thyroid-associated orbitopathy*, administration of Celecoxib blocks TGF-β-induced HA synthesis, while it decreases TGF-β- induced proliferation of ocular muscle fibroblasts (115).

- in addition, agonists of PPAR-γ (such as pioglitazone) modulate inflammation and induce adipogenesis in orbital fibroblasts in experimental cultures of human myoblasts from extraocular muscles from patients with *thyroid-associated orbitopathy* (115). These molecules inhibit TNF-a-mediated TGF-β-production through signalling pathways involving TGF-β/Smad or non-Smad–mediated signalling. Subsequently, TGF-β-induced differentiation of fibroblasts to myofibroblasts is inhibited and TGF-β, HAS and HA synthesis in retro-orbital myofibroblasts is decreased substantially, in a feed-forward paracrine action.

As a result in *thyroid-associated orbitopathy* COX2-inhibitors and PPAR-γ agonists can inhibit TGF-β-induced chemokine expression in cultured myoblasts from *thyroid-associated orbitopathy*, through signalling pathways involving TGF-β or Smad/non-Smad–mediated TGF-β signalling.

- in *thyroid-associated orbitopathy*, PAI-1-stimulated TGF-β synthesis could be blocked via functional inhibition of PAI-1 by natural or synthetic small molecules (already therapeutically applied in pathological conditions such as thromboembolic diseases, atherosclerosis, fibrosis and cancer) (116).

In conclusion, though no official TGF-β-associated treatments have been clinically approved for autoimmune thyroid diseases as yet, the direct and/or indirect enhancement or limitation of TGF-β immune -suppressive or -promoting activity, respectively, could be key to novel treatment strategies for these diseases (117). Such therapeutic interventions could result in reduced inflammation and apoptosis of thyroid epithelial cells, decrease of leucocytic infiltration of the thyroid gland, enhanced regeneration of damaged thyroid follicular cells, and inhibition of thyroid gland and orbital tissue fibrosis, all phenomena observed in these diseases. Because many TGF-β targeting approaches have already been successfully introduced for other autoimmune diseases and neoplastic conditions, it is of paramount importance to investigate the possibility of off-target effects in thyroid autoimmunity resulting from the therapeutic interference with with the physiology of such a ubiquitous molecule. The development of TGF-β based therapies is intriguing but has profound difficulties and dangers. It is unknown whether a long-term promotion or blockade of TGF-β signalling and activating pathways may result into off-target effects, because of the dual functions of TGF-β in the maintenance of tissue homeostasis. Thereby, crucial point for an effective and risk-free TGF-β targeted therapy, remains the intricate tuning of the signaling of this growth factor to the optimal magnitude in the right place at the right time. Further research is needed for the understanding of the insights into the interplay between TGF-β and the pathology and the development of thyroid autoimmunity.

5. Conclusions

TGF-β is possibly the most pleiotropic, multifunctional secreted growth factor known, one of its kind, as is produced by and act on a wide variety of immune and other cell types to regulate endpoints ranging from control of cell growth and differentiation to regulation of cellular function and targeting of gene activity. This growth factor, which mainly exerts suppressive but also under specific circumstances promoting immune activity, has attracted major research interest and efforts ever since its discovery thirty years ago. Unlike most other growth factors, that are ready to function upon secretion, TGF-β is unique in that it is secreted as part of a latent complex, that is stored in the extracellular matrix for activation and action at a later time point. Thereby the temporal and spatial activation of its ligand regulates and controls the magnitude and duration of TGF-β signaling. Under proper activation, TGF-β signaling has an essential role in immunity ranging from embryonic development to adult tissue homeostasis, whereas sustained activation or functional deletion *via* genetic mutations or environmental stimuli exacerbate its adversary effects and contribute to the pathophysiology of autoimmune thyroid diseases.

TGF-β is clearly a master regulator of the immune response, exerting either inhibitory or enhancing effects on cells of all arms of the immune system, which are highly relevant for the generation, development and possible therapeutic treatment of thyroid autoimmunity. Therefore, TGF-β has been described as “an excellent servant but a bad master”, in reference to its paradoxical characteristics, having the ability to exert both negative and positive effects on the immune system. The triggering and mediating factors, which ‘allow’ to TGF-β to perform such a broad spectrum of functions regarding thyroid autoimmunity are not fully understood and needs to be further studied.

The role TGF-β in the prognosis and prediction of autoimmune thyroid diseases is currently the focus of intensive scientific research, with hopeful results. Furthermore promising perspectives for the therapeutic use of TGF-β in case of thyroid autoimmunity exist. The main strategies include either supporting the immune-supressive role of TGF-β or blocking the pathways of signaling/activation and/or activity of this growth factor, depending on the specific autoimmunity type and pathophysiological stage. All these therapies face limitations, because of the possible dangers, hiding behind an uncontrolled stimulation and action or suppression and blockade of TGF-β. Thus, for systemic treatments involving the administration or inhibition of TGF-β, the precise balance between the suppression and/or promotion of immune system *via* immune cells should be achieved. In every case the development of the previous strategies are still in early research stage and further well controlled population studies and clinical trials are needed in order to establish the therapeutic value of TGF-β in thyroid autoimmunity.

Conclusively, a comprehensive understanding of the mechanisms related to the physiological or pathological effects of TGF-β, can unveil the whole spectrum of the TGF-β-mediated thyroid autoimmunity and lead to promising diagnostics and therapeutics based on TGF-β. Novel strategies that tune TGF-β signaling to properly respond to specific contexts should be developed with associated clinical implications on autoimmune thyroid diseases.

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9. Legends to the Figures

Figure 1: Immune mechanisms of *Graves' disease* and *Hashimoto's thyroiditis*. Both autoimmune thyroid diseases arise when various factors (endogenous, exogenous and genetic) precipitate thyrocyte destruction followed by autoantigen release. The latter are presented by APCs (Antigen presenting cells) to $CD4^+$ T lymphocytes. In *Graves' disease*, activated $CD4^+$ T cells induce B cells to secrete thyroid-stimulating immunoglobulins (TSI) against the thyroid-stimulating hormone receptor (TSHr), resulting in unrestrained thyroid hormone production and hyperthyroidism. In Hashimoto's thyroiditis, self-reactive $CD4^+$ T lymphocytes recruit B cells and $CD8^+$ T cells (CTLs) into the thyroid. Both cells, *via* different mechanisms lead to disease progression, resulting into the death of thyroid cells and hypothyroidism.

Figure 2. TGF-β production and activation. All TGF-βs are synthesized as precursor molecules (biologically inactive forms) containing a propeptide region in addition to the TGF-β homodimer. After it's synthesis, the TGF-β homodimer interacts with a Latency Associated Peptide (LAP), a protein derived from the N-terminal region of the TGF-β gene product, forming a complex called Small Latent Complex (SLC). This complex remains in the cell until it is bound by another protein called Latent TGF-β-Binding Protein (LTBP), forming a larger complex called Large Latent Complex (LLC). It is this LLC that gets secreted to the extracellular matrix (ECM). In most cases, before the LLC is secreted, the TGF-β precursor is cleaved from the propeptide but remains attached to it by noncovalent bonds. After its secretion, it remains in the extracellular matrix as an inactivated complex containing both the LTBP and the LAP which need to be further processed in order to release active TGF-β. Proteases, integrins, pH, and reactive oxygen species are some of the factors that contribute to TGF-β activation. Only following disruption of this binding, mature TGF-β is activated and can bind to its receptors.

Figure 3: TGF-β signalling pathways. Active TGF-β mediates its biological functions by binding to TGF-β receptors (TβRs). TβRIII (also called betaglycan) regulates/controls access of TGF-β to TβRI and TβRII, modulating thus, the intracellular TGF-β activity. In detail, TβRII (when bound on the cell surface), facilitates active TGF-β binding to TβRII, which is followed by TβRI recruitment. This results to a formation of a tetrameric TβR complex (two TGF-βRI and two TGF-βRII molecules). Following the formation of this tetramer, TβRs phosphorylate downstream regulatory Smads (2/3) [The abbreviation Smad refers to the homologies to the Caenorhabditis elegans SMA ("small" worm phenotype) and Drosophila MAD ("Mothers Against Decapentaplegic") family of genes] resulting to a conformational change which permits formation of a complex between Smads 2/3 with either Smad4 or TIF1γ (transcriptional intermediary factor 1γ). The

Smad2/3-Smad4 or Smad2/3-TIF1γ complexes subsequently translocate to the nucleus, where they target gene transcription in cooperation with other transription factors (TFs) and co-factors. Of note inhibitory Smads (6/7) (I-Smads) can obstruct the Smad-mediated signalling process. Alternatively, TGF-β activates various cell type-specific Smad-independent signalling pathways, including those mediated by TAK1 (TGF-β-mediated kinase 1), MEK or MKK (Mitogen-activated protein kinase kinase), JNK (c-Jun N-terminal kinases), P38 or MAR (p38 mitogen-activated protein kinases), PI3 (Phosphoinositide 3-kinases), Rho family proteins, and the Par6 (epithelial polarity protein 6).

Figure 4. Regulatory effects of TGF-β on T-cells. TGF-β is critical for the differentiation of CD4+/CD8+ to CD4+ (and further to Tfh-cells), CD8+, nTreg and NKT cells, a process taking place in the thymus gland. Furthermore, in the periphery, limits the apoptosis of CD4+ and CD8+ cells, thus leading to prolonged survival of the latter. On the other hand, TGF-β inhibits further proliferation/differentiation of Th1-, Th2- cells and CTLs. This transforming growth factor promotes nTreg survival, while it inhibits their proliferation. Lastly TGF-β plays a crucial role regarding iTreg- and Th17 differentiation.

Figure 5. The role of TGF-β in the pathogenesis of thyroid-associated orbitopathy (TAO). In TAO, orbital fibroblasts (OFs) are infiltrated by T-, B-cells and $CD34^+$ fibrocytes. OFs produce TGF-β which contributes to TSHr degradation and to adipogenesis deceleration. Furthermore TGF-β stimulates OFs' differentiation and proliferation via Hyalorunan Synthase-1 (HAS1) and -2 (HAS2) expression into cell-surface marker Thy-1 (CD90) expressing OFs, which reside into the extraocular muscles and produce increased TGF-β amounts. Under interaction with $CD4^+$ T-cells and TGF-β stimulation, these $CD90^+$OFs differentiate into myofibroblasts, which produce cytokines, leading to fibrosis and extraocular muscle enlargement. On the other hand, OFs can differentiate into orbital adipocytes via T- and B-cell regulation and TGF-β mediation, resulting into expanded adipose tissue. These orbital adipocytes are capable of TGF-β-mediated differentiation into mature adipocytes with increased TSHr expression. These cells, contribute to orbital fat expansion and indirectly to fibrosis via, among others, serine protease plasminogen activator inhibitor-1 (PAI-1) and Sphingosine-1-phosphate (S1P) mediation. Thus, TGF-β is critical for development of TAO and establishment of fibrosis in the latter.

10. TABLES

Figure 1. Immune mechanisms of *Graves' disease* and *Hashimoto's thyroiditis*

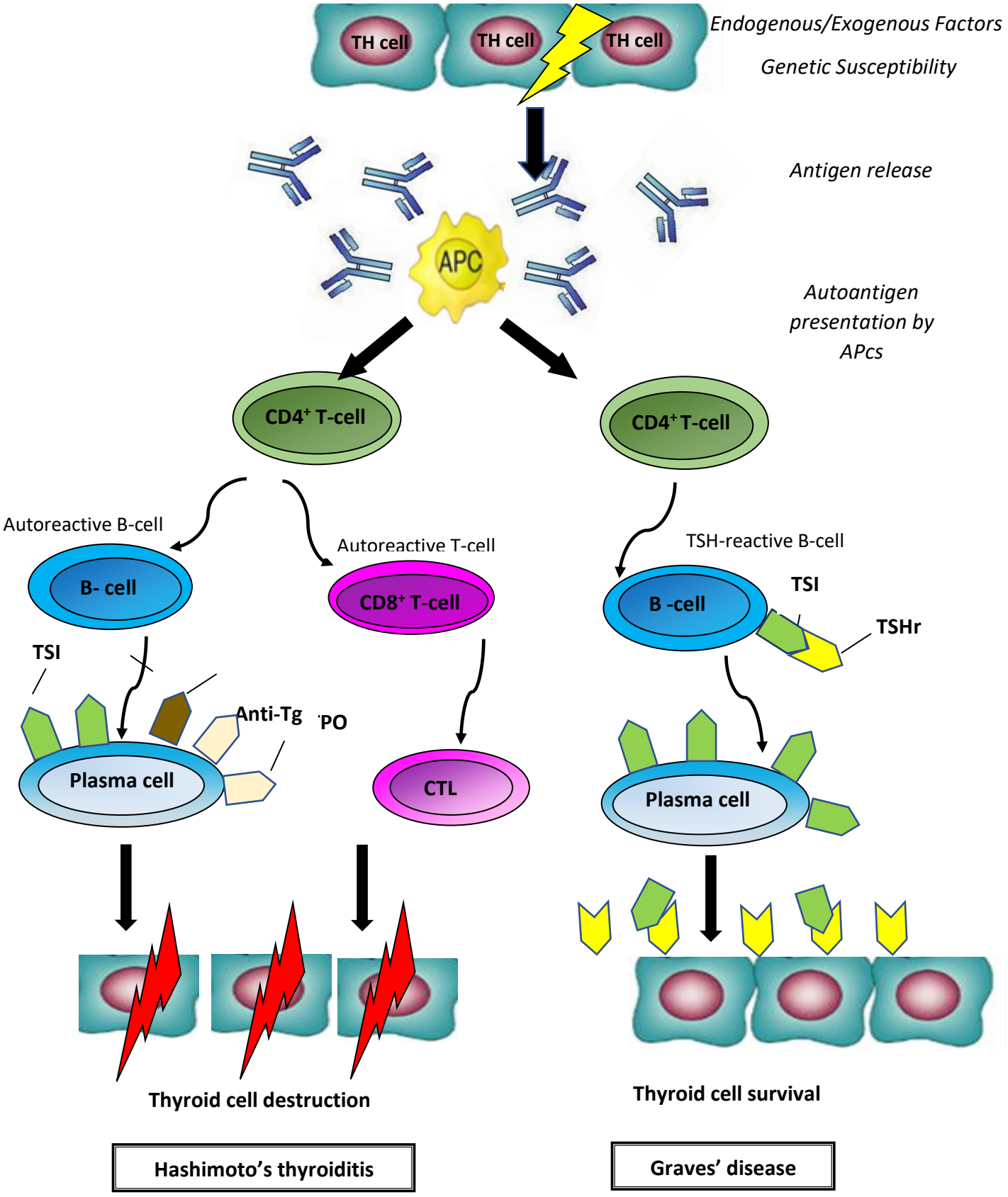

Figure 2. TGF-β production and activation

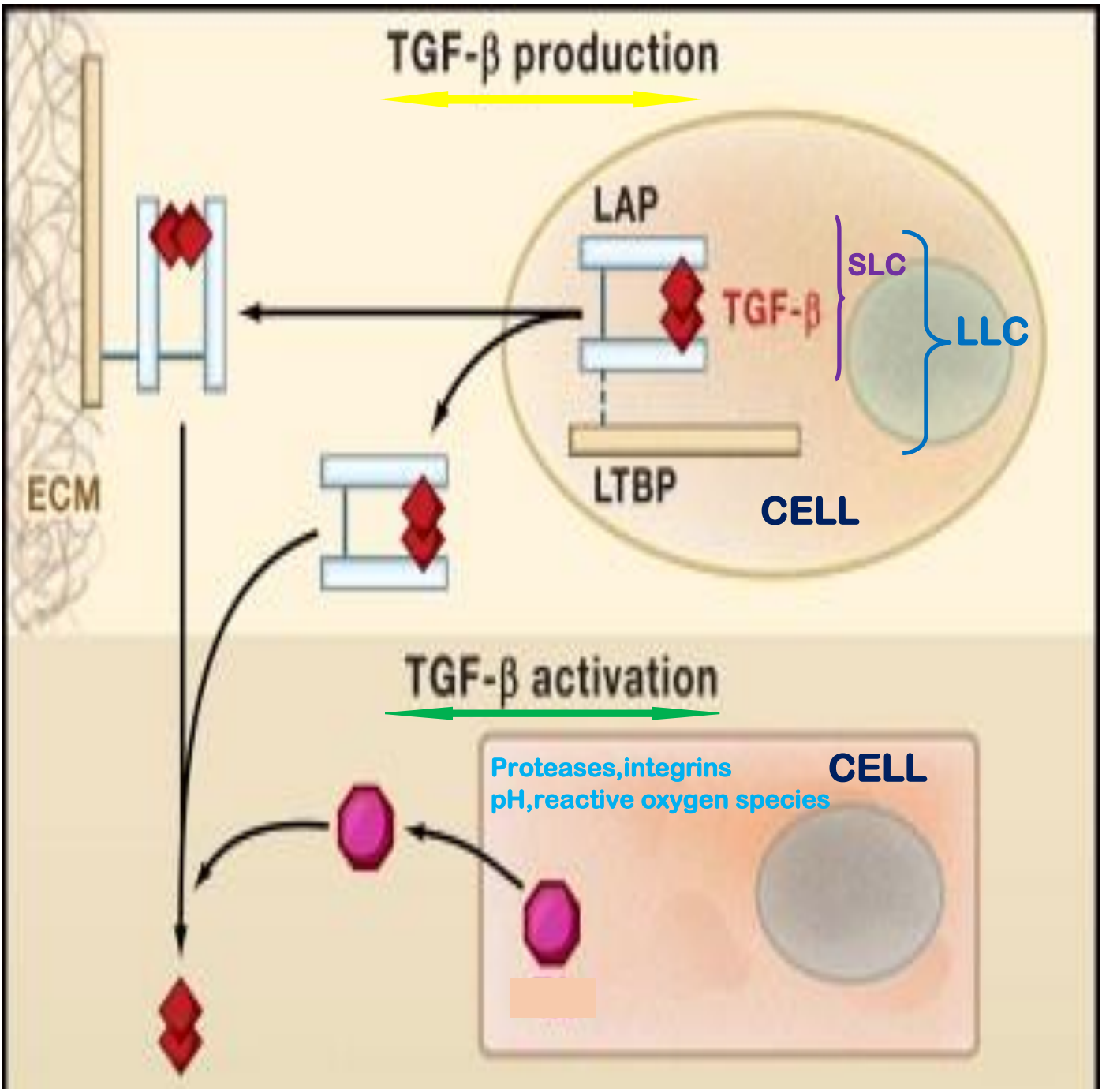

Figure 3. TGF-β signalling pathways

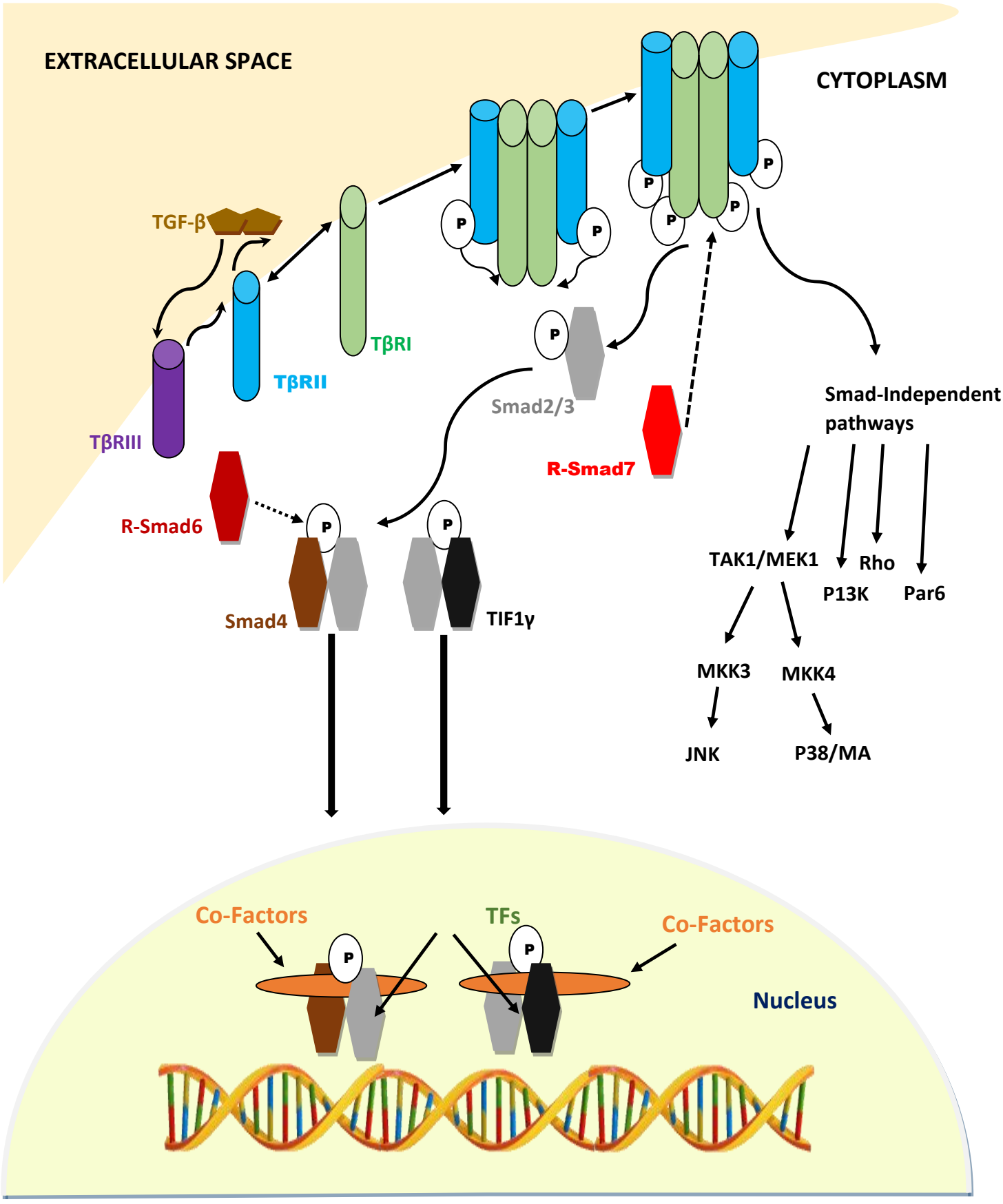

Figure 4. Pleiotropic effects of TGF-β on T-cell development, proliferation, and differentiation

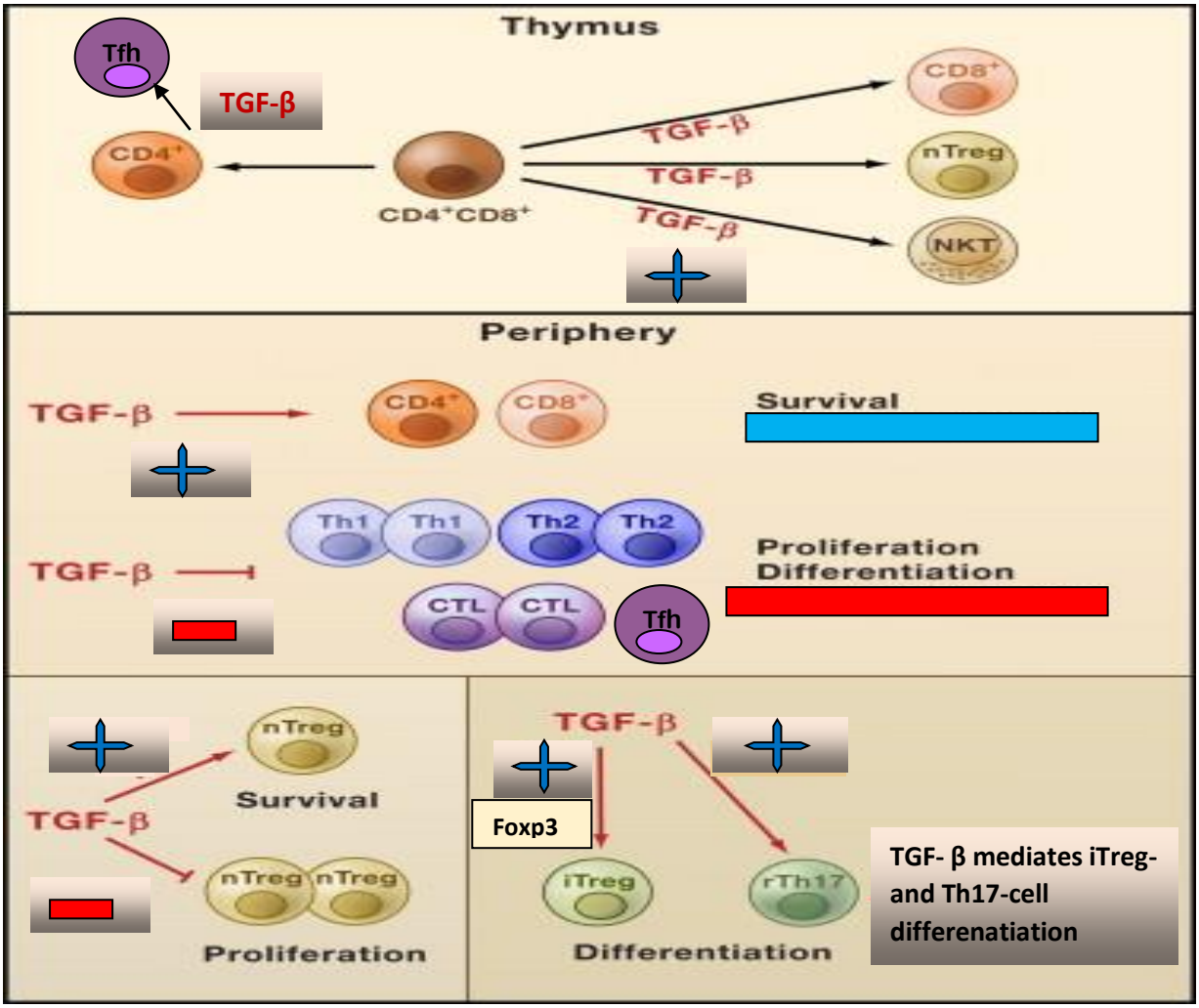

Figure 5. The role of TGF-β in the pathogenesis of thyroid-associated orbitopathy

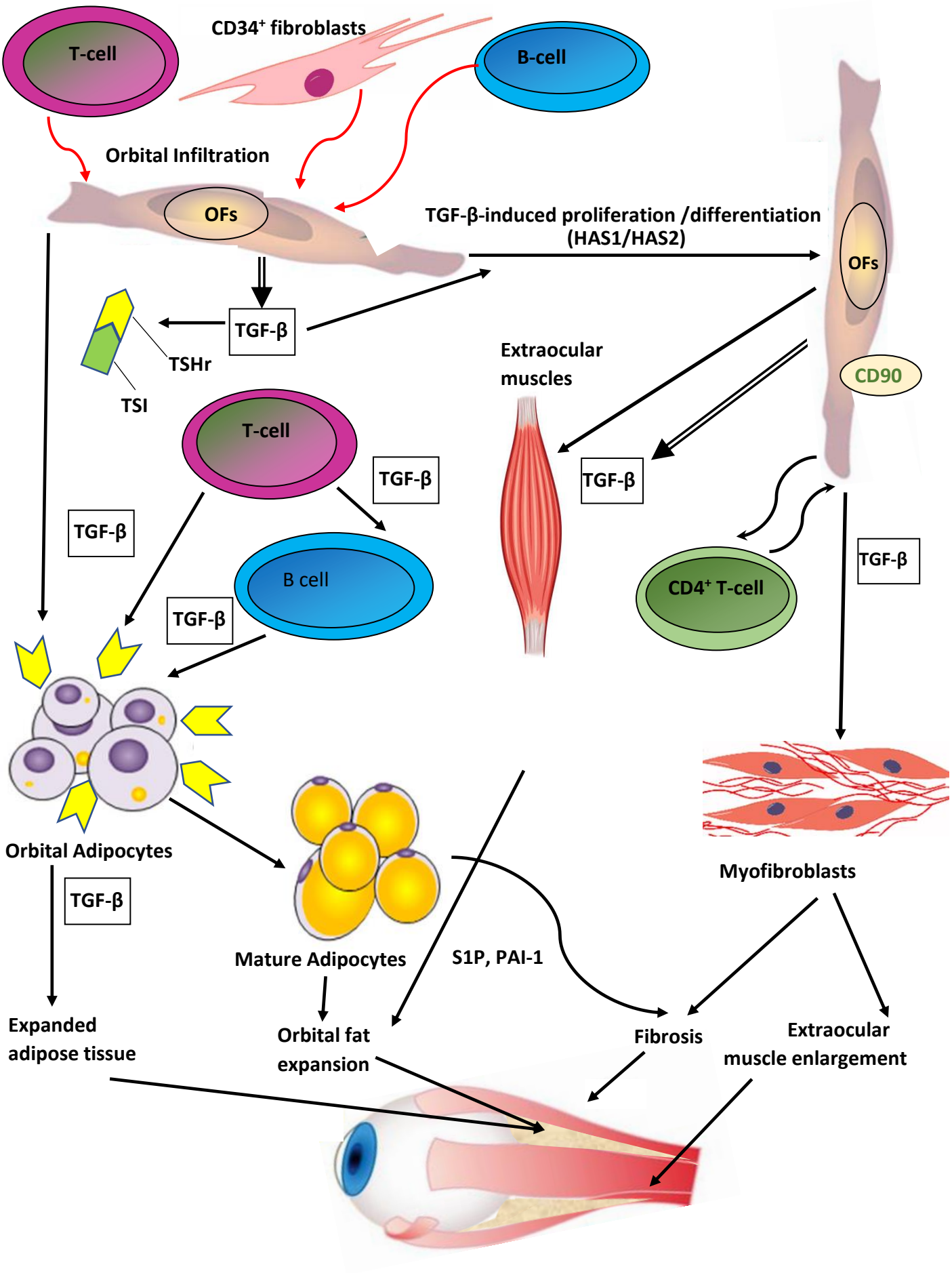

Table 1. Cell categories in *innate* immunity

- **Leucocytes**

  Natural killer cells (allias NK cells)
  - attack and destroy compromised host cells (tumor cells and virus infected cells), expressing low levels of MHC I
  - spare normal host cells

  Mast cells
  - reside in connective tissue and in the mucous membranes
  - are associated with wound healing, allergy, anaphylaxis and defence against pathogens
  - following activation, they rapidly release histamine and heparin and chemotactic cytokines initiating neutrophils and macrophages recruitment

  Eosinophils
  - upon activation, they mediate defence against parasite and regulate allergic reaction

  Basophils
  - following activation, they produce highly toxic proteins and free radicals, effective in killing parasites
  - cause tissue destruction during an allergic reaction

- **Phagocytic cells**

  Macrophages
  - are large phagocytic leukocytes, which are able to move outside of the vascular system
  - In tissues, organ-specific macrophages are differentiated from phagocytic cells present in the blood called monocytes
  - are the most efficient phagocytes and can phagocytose bacteria or other cells or microbes

  Monocytes
  - are bone marrow-derived immune cells
  - migrate to sites of injury in inflammation
  - regulate phagocytosis, secretion of cytokines, and antigen presentation
  - differentiate into tissue macrophages

  Neutrophils (allias granulocytes or polymorphonuclear cells)
  - are the most abundant type of phagocytes, normally representing 50-60% of the total circulating leukocytes
  - are large phagocytic leukocytes, which are able to move outside of the vascular system
  - are usually the first cells to arrive at the site of an infection
  - produce toxic substances that attack pathogens and kill or inhibit bacteria and fungi growth

  Dentritic cells (allias DCs)
  - are derived from common dendritic cell progenitors in the bone marrow
  - are subdivided into conventional and plasmacytoid dentritic cells as well as a specialized population of dentritic cells called Langerhans cells, which reside in the skin
  - populate almost every tissue
  - initiate and direct the immune response
    - sense danger signals, sample antigen, and migrate to T cell-rich areas in lymphoid tissue to present antigens to initiate a specific T cell response
    - serve as a link between the innate and adaptive immune systems

- **Innate-like T-cells (allias γδ T-cells and NK-T-cells)**
    - develop and differentiate from bone marrow-derived lymphoid stem cells in the thymus
    - bear invariant T cell receptors (TCRs)
    - they are part of the pattern recognition receptor
- **Innate lymphoid cells**
    - are a distinct, lineage-negative group of cells within the lymphoid arm of the innate immune system
    - based upon their transcriptional profiles, they are split into three groups: Innate lymphoid cells 1, 2 and 3, which express different transcription factors and produce various cytokines

Table 2. T-cell categories in *adaptive* immunity

CD4+ cells (*allias* helper T-cells or Th-cells)

- leading role in cellular immunity and contribute to humoral immunity
- classified in Th1, Th2, Th9, Th17, Th22 and Tfh (follicular helper) T-cells, according to the type of cytokine they produce and their role in immune defence and autoimmunity

Cytotoxic $CD8^+$ T-cells (*allias* CTLs)

- vital role in destruction of virus-infected cells and tumor cells
- associate with MHC class I molecules and produce IL-2 and IFN-γ

Memory T-cells

- derived from naïve T-cells when MHC molecules on APCs encounter an antigen
- classified in central memory, effector memory, tissue resident memory and virtual memory T cells

T regulatory cells (Tregs or suppressor T-cells)

- derived from CD4+ cells)
- classified in natural Tregs (*allias* nTregs or $CD4^+CD25^+$ T-cells) and adaptive (induced) Tregs (*allias* iTregs or $CD4^+CD25^-Foxp3^+$)

Effector T-cells (Tef-cells)

- derived from CD4+, CD8+ or Treg cells
- actively respond to a stimulus

Table 3. Characteristics of thyroid autoimmunity based on its type

	Thyroid status/function	Typical signs/symptoms	Immune/ genetic factors involved	Type of immune reaction	Result of immune reaction	Stages/Evolution
Grave's disease	most common cause of hyperthyroidism	hyperthyroidism goiter thyroid-associated ophthalmopathy pretibial myxedema	increased TRAbs thyrotropin receptors THSrs $CD4^+$ cells B-cells\genetic factors	humoral and cellular immune mechanisms	stimulation of thyroid hormones synthesis and secretion follicular cell growth thyroid gland hyperplasia (diffuse goiter)	thyroid cell survival increased thyroid gland function Th 2 pattern of cytokine production increased TGF-β concentrations
Hashimotos' thyroiditis	most common cause of hypothyroidism	profuse lymphocytic infiltration lymphoid germinal centres destruction of thyroid follicles decreased thyroid gland function	increased anti-TPO and/or anti-Tg auto antibodies MHC class II antigen-presenting cells (APC) $CD4^+$ /$CD8^+$ /B cells genetic factors	humoral and cellular immune mechanisms	gradual thyroid destruction, with (goitrous autoimmune thyroiditis) or without (atrophic autoimmune thyroiditis) goiter formation	Th1 pattern of cytokine production initial stage: lower TGF-β concentrations final stage: increased TGF-β concentrations – fibrosis
Thyroid-associated orbitopathy	affects extraocular muscles and surrounding orbital connective tissue can occur in Grave's disease (mainly) but also in Hashimotos' thyroiditis	upper eyelid retraction periorbital oedema proptosis lagophthalmos impaired eye motility protrusion optic nerve compression	TSHrs found on thyroid cells TRAbs autoimmune cross-reactivity against shared antigens between the thyroid cells and retro-orbital tissues $CD4^+$ /$CD8^+$ /B cells genetic factors	humoral and cellular immune mechanisms	infiltration of orbital muscular cells (Ofs) and fibroblasts by T-cells, mast cells and plasmatic cells Ofs' proliferation and differentiation into myofibroblasts and adipocytes production of glycosaminoglycans chemokines and cytokines	the early stage is caused by cell-mediated (Th1-type) immune response the late stage involves a humoral-mediated (Th2-type) immune increased TGF-β concentrations extraocular muscle enlargement orbital fat expansion fibrosis
Thyroid disease during pregnancy	commonest are Grave's disease and Hashimoto's thyroiditis	abnormalities of thyroid gland function	Anti-TPO auto-antibodies $CD4^+$ cells $CD8^+$ cells B-cells/genetic factors	humoral and cellular immune mechanisms	decreased Th2- and increased Th1- and B-cell- associated immunity decreased maternal cortisol concentrations	dereased TGF-β serum concentrations in the 2^{nd} trimester compared to the 3^{rd}
Post-partum thyroiditis	destructive autoimmune thyroiditis, variant of *Hashimoto's thyroiditis* manifests within the first year following parturition	usually painless development of transient thyrotoxicosis and/or hypothyroidism	Anti-TPO and less anti-Tg auto-antibodies $CD4^+$ cells $CD8^+$ cells B-cells	humoral and cellular immune mechanisms	widespread thyroid cell lysis excessive amounts of thyroid hormone (toxic phase), followed by a resulting thyroid cell loss (hypothyroid phase), thyroid cell regrowth, and thyroid function recovery	Increased TGF-β concentrations Presence of other autoimmune diseases associated with increased risk of post-partum thyroiditis some women develop permanent hypothyroidism or goiter

Table 4 . Effects of serum TGF-β concentrations alterations on autoimmune thyroid diseases

	Graves' disease	**Hashimotos' thyroiditis**	**Thyroid-associated orbitopathy**	**Autoimmune thyroid disease during pregnancy**	**Post-partum thyroiditis**
Increased TGF-β concentrations	Can be observed in cases of severe TAO, related to enhanced limitation of TGF-β immunosupressive role	are associated with increased fibrosis in its final pathophysiologic phase	incite myofibroblast differentiation and cytokine production accelerate ocular fibrosis, tissue remodelling and matrix expansion correlate with its severity	observed in the third trimester, are related with increased risk of post-partum thyroiditis development	mark its hyperthyroid phase
Decreased TGF-β concentrations	contribute to the etiopathogenesis and the development of Graves' disease are associated to greater severity and susceptibility to this disease	facilitate its initial pathophysiologic stages are associated to the severity of this disease		are associated with limited suppression of thyroid autoimmunity lead to insufficient immune tolerance of the fetus	are associated, in its euthyroid phase, with increased concentrations of anti-TPO antibodies correlate with promotion and increased intractability

Table 5. Therapeutic interventions based on level of TGF-β synthesis, activation, and action with potential use on thyroid autoimmunity

	TGF-β				
	Effect on TGF-β serum concentrations	Effect on synthesis	Effect on activation	Effect on action	Therapeutic application
iTregs	Increase	Positive effect	None	None	Multiple sclerosis
Progesterone	None	None	Prevents TGF-β-induced Smad2/3 activation Inhibits expression of TGF-β/Smad2/3-induced genes	None	Bronchopulmonary dysplasia
Androgens	None	None	Represses binding of Smad3 to SBE	None	
Genetic transfer of Smad7	None	None	Inhibits Smad2/3 phosphorylation	None	Diabetic kidney disease, alcoholic steatohepatitis
Pentoxyphilline	Decrease	Down-regulates TGF-β expression	None	None	Glomerularproteinuric nephropathy, sarcoidosis
Antisense oligonucleotides/ antisense RNA	Decrease	Suppresses TGF-β production			Neoplastic conditions
Small peptides	None	None	Inhibits TGF-β disengagement from LAP	None	
Monoclonal anti-TGF-β	None	None	None	Neutralizes excess extracellular TGF-β	Animal models of Hashimoto's thyroiditis
Triiodothyronine/nuclear receptor ligands	None	None	None	Limits Smad phosphorylation	Animal models of thyroid fibrosis

Estrogen receptor β antagonists	None	None	None	Iinhibits TGF-β mediated Th17-type response	Animal models of experimental thyroiditis
Estrogen receptor agonists	None	None	None	Suppression of TGF-β activity	Animal models
COX-2 inhibitors	None	None	None	Blocks TGF-β induced HA-synthesis Decreases TGF-β induced ocular muscle fibroblasts proliferation	Human cultures of TAO extraocular muscle fibroblasts
Agonists of PPAR-γ	Decrease	Inhibits TNA-a mediated TGF-β production	None	Inhibits TGF-β Induced differentiation of fibroblasts to myofibroblasts Decreases HAS and HA synthesis	Human cultures of TAO extraocular muscle fibroblasts
PAI-1 inhibitors	Decrease	Inhibits TGF-β synthesis	None	None	Thromboembolic diseases, Atherosclerosis