Central Tolerance Mechanisms to TSHR in Graves’ Disease: Contributions to Understand the Genetic Association

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ABSTRACT
In the last 3 years, the association of thyrotropin receptor gene (TSHR) variations to Graves’ disease (GD) has been confirmed. It is now well established that a 30 Kb region of intron 1 of the TSHR gene is linked to GD predisposition. Elucidating the mechanism(s) by which these polymorphisms confer susceptibility is difficult but would constitute an important advance in endocrine autoimmunity in general. Two hypotheses, both postulating TSHR gene regulatory mechanisms, are discussed. One postulates differential level of expression in the thymus, involving central tolerance. The other postulates a shift in TSHR differential splicing leading to the production of soluble proteins that will have easy access to antigen presenting cells, so it is focused in peripheral tolerance. A combination of the 2 hypotheses is feasible, especially under the light of recent evidence that have identified epigenetic factors acting on TSHR intron 1.

Introduction
Since we wrote a similar review on genetics of Graves’ disease for Hormone and Metabolic Research 3 years ago [1], the association of thyrotropin receptor gene (TSHR) variations to Graves’ Disease (GD) has been further confirmed and additional comprehensive reviews have been published including extensive meta-analyses [2–5]. It is well established that a 30 Kb region at the 5′ end of the intron 1 (a large intron of 106 Kb) of the TSHR is linked to GD predisposition.

The attention is now focused in trying to understand the mechanism(s) by which these polymorphisms confer disease susceptibility. As in other areas of autoimmunity and of polygenic diseases in general, investigators are tackling the missing heritability riddle, that is, the difference between observed heredity and heredity attributable to identified gene variations. Several lines of investigation are being followed, one of them being the influence of epigenetic mechanisms. Indeed, one of the mechanisms proposed to explain TSHR variation and GD involves epigenetic regulation [6]
Central Tolerance to Thyroid Autoantigens

The first suggestion that central tolerance was based on deletional mechanisms in the thymic gland during T cell generation dates back to Jacques Miller’s work in the 1970’s that revealed the role of this gland as a primary lymphoid organ (reviewed in [7]). The analysis of thymus anatomy and the high rate of T cell death led to this proposition. In 1980’s, the works of Marrack and Kappler [8] and von Boehmmer [9] groups provided the first clear experimental evidence for the negative selection of self-reactive thymocytes. However, thymic negative selection of developing autoreactive thymocytes seemed to apply only to those thymocytes whose TCR would recognised antigens found in the thymic microenvironment. Because this gland contains both cells of hematopoietic lineage plus various sublineages of epithelial cells, the offer would be wide enough to ensure general tolerance even if not comprehensive of all proteins in the body. On the other hand, it is known since the 70’s that circulating proteins can diffuse into the thymus medulla and that the concept of “blood-thymus” barriers applies only to the thymus cortex [10, 11]. In addition, antigens transported by dendritic cells to the thymus can also be involved in negative selection [12, 13]. These would contribute to maintaining tolerance to innocuous non-self-antigens such as those derived from food or the microbiome. Interestingly, traffic of dendritic cells to the thymus is restricted during infection, likely as a protective mechanism. Therefore, the offer of self-antigens in the thymus for negative selection did not include, according to the evidence available in the 1980’s, the tissue restricted antigens (TRA) or tissue specific antigens (TSA), a category to which endocrine self-antigens belong. Thus, it was assumed that tolerance to thyroglobulin (TG), thyroid peroxidase (TPO), thyrotropin receptor (TSHR) and other less well characterised thyroid-specific proteins relied on unspecified peripheral tolerance mechanisms.

However, by the 1980’s there was already some evidence that peripheral antigens not expected to be expressed in the thymus, were actually expressed. The first clue was the work by the oncology group of Douglas Hanahan that detected tolerance to SV40 large T antigen expressed transgenically under the control of the rat insulin promoter (RIP). This promoter should have ensured that the T antigen was only expressed by the islet beta cells and consequently it would not generate immunological tolerance. But, surprisingly, there was tolerance to the T antigens and when investigated, the large T antigen was detected in the thymus in addition to the islets; moreover, RNA for insulin itself was also detected in the thymus of non-transgenic mice of the same strain [14]. Similar observations were made in other transgenic systems in which cell lineage-specific promoters resulted in thymus expression of the transgenes and central tolerance [15]. After ruling out artefacts dependent of the integration site of transgenes in the mouse genome, these findings lead to the conclusion that a special mechanism was operating in the thymus that determined the expression of some peripheral self-antigens [16].

The suspicion that high-affinity T cells specific for thyroid and pancreatic islet antigens did not reach the periphery arises, in our case, from the low responses to self-antigens by auto-reactive T cells clones obtained from autoimmune tissue samples (GD thyroid and type 1 diabetes pancreas). Responses were orders of magnitude weaker than those obtained with recall antigens or in allogeneic systems [17–21]. There were 2 main possible explanations for these weak responses: 1) Regulation, that is, we were dealing with T cells that were partially anergic. This was not likely because after days in culture with antigens, APCs and IL-2, classical anergy should be overcome and it did not; 2) Affinity, that is, we were dealing with low affinity T cells. The most likely explanations for this low affinity were that high affinity clones had been deleted and the place to look for deletion was the thymus. We therefore investigated the expression of peripheral antigens in the thymus, as this was the essential requirement to induce their deletion. Quantitative PCR was not yet widely available and we resorted to carefully calibrated nested radioactive PCR. The results were very clear: TG, insulin, GAD67, TPO, myelin basic protein and retinal S antigen were all expressed in glands from 8 days to 12 years-old donors. Cell separation experiments indicated that the mRNAs for these antigens were associated with the stromal epithelial cells and not with thymocytes [22]. At that time, 2 groups produced evidence indicating that the level of insulin transcription in the thymus was linked to polymorphisms present in the promoter of the insulin gene (a VNTR element). They proposed that the long-time known association of the insulin gene with type 1 diabetes was due to the influence of this polymorphism on the level of insulin expression in the thymus [23, 24]. Therefore, these authors did not only confirm the unexpected expression of insulin in the thymus, but they linked predisposition to T1D with the level of insulin expression.

The expression of a broad selection of peripheral antigens in the thymus was finally described in detail by the group of Bruno Kyewski who proposed to name it “promiscuous gene expression” (PGE) [25]. In their paper, the only thyroid antigen investigated was TG, which was expressed at high levels, but not exclusively, by medullary thymus epithelial cells (mTEC). Other endocrine antigens, that is, insulin, GAD65, GAD67, and I-A2, all islet antigens, were also expressed by mTECs. Some organ specific antigens such as retinal S antigen, and some acute phase reactants such a serum amyloid P component and C-reactive protein were also expressed by mTECs. They also demonstrated the presence of the corresponding protein in a low number of mTECs, in the order of 0.5–5 % depending on the antigen. During the last 20 years thymus PGE has been extensively demonstrated at mRNA level [25–29], but it was not until year 2015 when our group reported for the first time the presence of peptides derived from TRAs within the HLA-DR-associated human thymus peptidome [30].

In parallel, groups working on the identification of the gene causing a rare syndrome called Autoimmune Polyendocrinopathy with Candidiasis and Ectodermic Dysplasia (APECED) or APS1 (Autoimmune Polyendocrine Syndrome type 1) shed light on this “Promiscuous Gene Expression” phenomenon of the thymus. APECED is an autosomal recessive disease in which patients develop multiple autoimmune diseases, typically primary hypoparathyroidism, Addison’s disease, autoimmune gastritis, alopecia, hypogonadism, but they also suffer from chronic candidiasis and dental enamel...
dysplasia. Positional cloning lead to the identification of a gene that was designated AIRE (for Autoimmune REgulator), which had features of a transcription regulator and was almost exclusively expressed by the thymus epithelial cells [31, 32]. The circle was closed in 2002 when 2 groups [33, 34], showed that aire−/− mice developed an autoimmune syndrome affecting many organs, reminiscent of human APECEED. It has been later demonstrated that AIRE is also expressed at low levels by dendritic cells of the lymph nodes, where it seems to improve their efficiency as APCs but does not induce promiscuous gene expression [35, 36]. All these findings brought the research focus of autoimmunity to central tolerance. Failures at this level had been dismissed as a cause of autoimmunity for decades because the thymus was not supposed to be involved in tolerance to tissue-restricted antigens, such as the targets of endocrine autoimmune diseases. The mechanism through which AIRE regulates promiscuous gene expression is still an area of very intensive investigation [37, 38]. An additional factor promoting PGE, FezF2, has been recently identified and work is in progress to establish its contribution to central tolerance [39, 40]. Rather counterintuitively, polymorphisms in AIRE itself are not associated to GD nor are frequent cause of autoimmunity [41].

As mentioned above, the expression of thyroid antigens in the thymus was investigated in our laboratory as part of a project aimed at understanding the role of central tolerance in thyroid autoimmunity and we concluded that TG was expressed at a high level by thymus epithelial cells (mTEC > cTEC) while TPO was expressed at low level by the AIREhigh mTECs, with marked individual variation. Yet, the role of TPO and TG as pathogenic autoantigens is not yet fully established, although they are both targets of thyroid autoimmunity-related autoantibodies. Paradoxically, thymus expression of TSHR – a definitely pathogenic antigen – had not been investigated in this context, even if back in the 90’s some researchers demonstrated the expression of TSHR in the thymus and attributed thymic hyperplasia found often in GD patients to a local effect of TSHR Abs [42].

**TSHR Allelic Variation and Genetic Predisposition to Graves’ Disease**

TSHR gene is a 191 Kb gene located at chromosome 14q31. It has 10 coding exons and, in addition to the full-length canonical form, there are 2 additional main transcribed isoforms known as ST4 and ST5. These isoforms lack the large exon 10 that codes for part of the hinge region, transmembrane and cytoplasmic domain. Isoforms ST4 and ST5 are coded by the initial 8 exons plus an alternative exon 9. There is good evidence of transcription at nearly the 50 % level of the full length but the proteins have not been investigated in detail [43] (Fig. 1, ▶ 2).

We decided to assess in detail the level of TSHR expression in the thymus but first we carried out a case control association study with 54 SNPs mapping in the TSHR gene in Graves’ disease (n = 137) vs. controls (n = 192). The results were clear and pointed to a SNP in intron 1 (rs179247) as the most closely associated (OR 2.42) [44]. By the time we got these results, the group of Stephen Gough had already reported very similar results in a large series of over 1000 GD patients and 900 controls from the UK [45, 46]. As the authors stated, TSHR was the major thyroid specific gene associated to Graves’ disease and this definite results constituted a milestone after years of contradictory reports on the association between TSHR and GD.

The genetic association studies rarely provide information on the mechanism by which a given polymorphisms confer susceptibility to disease. Most disease-associated SNPs are located in non-coding regions and it is assumed that they act by influencing gene expression. SNP 179247 is located 10 Kb 3’ to the end of exon 1 within the 106 Kb intron 1 of the TSHR (Fig. 1), and no explanation on its function was suggested from the analysis of sequence motifs in the regions flanking this SNP. Before considering the different hypotheses that are discussed in the literature to explain this association, we will review briefly some unexpected features of TSHR expression.

**TSHR Expression in the Thymus, Cell Distribution, and Implications for the Differential Effect of Intron 1 Alleles**

TSHR is a gene expressed in the thyroid at moderately low levels. Recent data from RNAseq analysis of 446 glands gave an average of 200–500 transcripts per million (TPM) that is approximately 50 % of the expression level of GAPDH (average 800 TPM), a housekeeping gene widely used as reference in gene expression analysis, and certainly much below TG at 8000 TPM (www.gtexportal.org). There are no data on TSHR expression in the thymus in this or other open transcriptomic databases but our own data indicate that the level is approximately one fourth the level observed in thyroid (Fig. 3); this level is well above that of insulin (INS) or H+/K+ ATPase (ATP4A) that are AIRE dependent genes expressed by mTEC through PGE. Such relatively high level of expression pointed to a functional expression rather than to PGE. TSHR expression was demonstrated initially by qPCR in total tissue and later in thymus cells fractions [44]. Western blotting analysis confirmed thymus expression at a level not much below the thyroid’s, but this was probably exaggerated because of normalisation by protein content rather than by number of cells (in thyroid total protein content, colloid proteins dilute cellular proteins) [47].

In accordance with its levels of expression, TSHR in thymus is barely present in mTECs but much more expressed by maturing thymocytes where it seemed to be involved in differentiation expansion regulation, as suggested by extensive experiments with human thymus cultures and in a tshr−/− mouse model [48]. TSHR was found to be expressed from early thymic progenitors to double positive thymocytes, quickly lost in mature single positive thymocytes and totally absent in peripheral T cells, even in the recent thymic emigrants fraction [47]. In these experiments thymocyte cultures responded to human TSH and to monoclonal and patients’ polyclonal antibodies to TSHR with a clear increase of cytoplasmic cAMP, thus demonstrating the signalling capability of the receptor [47].

The above findings may contain the elements to start to understand why in Graves’ disease there is this unique tendency to generate stimulating autoantibodies to the TSHR, an exceptional type of autoantibody among the large variety of antibodies generated in the context of autoimmune diseases. As we have postulated it is conceivable that stimulating antibodies to the TSHR may result...
from the iterative boost of initially low affinity TSHR antibody producing B lymphocytes by TSHR specific T clones [1].

The expression of TSHR by thymocytes at relatively high levels but not by mTECs, the bona fide cell responsible for PGE and for negative selection, poses the question of how can TSHR induce central tolerance. The same question applies to lymphoid tissue antigens that are not specifically expressed by mTECs. It can be assumed that given the high number of thymocytes that die in the thymus as a consequence of the lack of TCR signalling during the early stages of their development (death by neglect), they should constitute a source of antigens that may be processed and presented by the macrophages that dispose of them.

If this is the case, one question that also arises is which form(s) of TSHR are more readily processed and presented in the thymus. This is an important question because one established mechanism of central tolerance failure is the expression of different isoforms of a protein in the thymus and in the periphery. This was first demonstrated by Klein and Kyewski in experimental acute encephalomyelitis induced by the myelin proteolipid protein (PLP) in SJL/J mice [49]. In this model, the autoimmune response was directed to peptides coded by exon 3 that is skipped in the isoform of the protein produced in the thymus, presumably by PGE. Since the original description, this has also been demonstrated for the islet antigen I-A2 [50] and is invoked to explain the autoantibodies to post-translationally modified proteins such as citrullinated peptides in rheumatoid arthritis [51]. It is conceivable that isoforms ST4 and, to a lesser extent, ST5, lacking the transmembrane domain, would be secreted and more readily available to induce negative selection than membrane-anchored TSHR [52]. In fact, recent results from our laboratory indicate that the shorter forms of TSHR, ST4, and ST5, are transcribed in the thymus at levels that, being soluble, can outcompete the complete protein, to provide peptides for negative selection (Marin-Sanchez et al., submitted). The consequence would be incomplete tolerance or “split tolerance” to a good portion of the hinge and the whole of the transmembrane domains of TSHR. In mice, TSHR isoforms have not been described and it is not known whether TSHR is expressed by the mTECs, but TSHR expression is much less tissue restricted than in humans. Therefore, it is possible that split tolerance to TSHR does not occur in mice and other placental mammals and this could explain why GD is a purely human disease. In fact, in mice, tolerance to TSHR is very solid and investigators had to resort to the generation of TSHR−/− mice [53], or to use very intense immunisation protocols to elicit a response to the TSHR [54]. This response only results in a very mod-
erate thyroid lesion and minimally detectable effect on thyroid function and some degree of Graves' ophthalmopathy. TSHR protein has been subjected to intensive analysis but until now it has not been possible to obtain a crystal of the full length TSHR. There are good resolution crystals of part of extracellular domain bound to stimulating (M22) and blocking (KI-70) human monoclonal antibodies [55, 56]. While the residues contacted by the TSH and these model autoantibodies have been defined [57], the T cell epitopes are not yet so well defined [58–60]. Inaba et al. identified a DR3-restricted 133–150 peptide that elicited significant responses in humans [60, 61], which almost coincides with the 142–161 peptide identified in DR3-transgenic mice immunised with an adenoviral vector containing the TSHR A subunit [62]. Yet evidence of an expansion of TSHR-peptide specific T cells using tetramer technology, as available in Type-1 diabetes [63, 64], has not been yet generated.

Two Hypothesis to Explain how Allelic Variants of TSHR Predispose to Disease

We tested the hypothesis that the SNP rs179247 might influence the level of expression of TSHR in the thymus by measuring the levels of TSHR expression in the thymus of individuals carrying each of the 3 possible allele combinations of the associated SNP. The results were remarkable; not only the thymic expression of TSHR was significantly lower (26.3 %), but the age of disease onset was also lower (29.8 ± 10.5 vs. 39.0 ± 10.5) in the carriers of high-risk alleles. On the other hand, SNP 179247 had no effect on TSHR expression levels in the thyroid gland. Other TSHR SNPs not associated to GD had no influence of TSHR thymus expression. So, we proposed that SNP rs179247 predisposed to GD by reducing the efficiency of central tolerance to TSHR because of its lower expression in the thymus [44].

In the paper by Brand et al., Gough’s group proposed a different but not excluding mechanism to explain the association [46].
According to it, SNP rs179247 would influence the proportion of TSHR gene transcripts in such a way that the predisposing allele would favour the transcription of the shorter ST4 and ST5 transcripts over the full length. ST4 and ST5 code for the initial 231 aa of 431 aa extracellular portion of TSHR plus a short sequence of 22 and 38 aa, respectively, coded by alternative exons included in the 31 Kb of intron 8, and would presumably be secreted (no experimental evidence). TSHR stimulating antibodies pathognomonic of GD bind to epitopes in this extracellular portion of TSHR. These authors postulated that the smaller proteins resulting from the translation of ST4 and ST5 mRNAs would be more immunogenic and accessible to the immune system than the full membrane-anchored TSHR [65]. This is supported by findings in an animal model of hyperthyroidism induced by an adenoviral vector [66].

The above hypothesis does not address the mechanism through which these SNPs modulate transcription. A more recent publication takes an epigenetic approach to it, and its conclusions would be applicable to both hypotheses [6].

Epigenetic influence on the heredity of GD should be taken into account having in mind that twin concordance is around 30 % [67], whereas the contribution of all known GD-associated loci can be estimated from their combined odd ratios to be around 10 %. This difference, better studied in traits like stature, has been designated as “missing heritability” [68]. Transgenerational inheritance of epigenetic traits – a field still in its infancy in mammals [69] – could play a role in this missing heritability and it could be applicable to autoimmune thyroid diseases [70].

In a very interesting study, Stefan et al. investigated whether the TSHR intron 1 region containing the SNPs associated to GD could regulate gene expression through an epigenetic mechanism [6]. It should be noticed that inside the large intron 1 (106 Kb), the region containing GD-associated SNPs has approximately 30 Kb. Their analysis showed that there is a subregion centred around SNPs rs12101255 and rs12101261 (separated by only 177 bp) that is within an open chromatin area. The authors demonstrated that this subregion binds the transcriptional repressor PLZF that is under the control of IFNα. The predisposing allele binds more efficiently the PLZF repressor factor, leading to lower TSHR expression. These authors also confirmed that the TSHR intron 1 alleles predisposing to GD show lower expression in the thymus. In favour of the hypothesis is the confirmed observation that type 1 interferons, when used in therapy for hepatitis C, can trigger autoimmune thyroiditis in some patients [71, 72]. Moreover there is a clear IFN expression signature in the transcriptomic profile of GD tissue [73], confirmed by RNAseq [6]. Finally, viral infection is one contemplated trigger of thyroid autoimmunity. These experiments were conducted in thyroid follicular cells in culture and reporter system and therefore the demonstration that IFNα in the thymus has an allele dependent effect on TSHR expression is still missing. It is however conceivable that during infection, circulating IFNα reaches the thymus and reduces TSHR expression, thus favouring the escape of TSHR reactive T cells. The authors do not speculate on whether this epigenetic effect could be trans-generationally inheritable. Interestingly, a recent paper reported numerous methylation and histone acetylation differences among patients and controls in genes involved in TCR signalling regulation and in intron 1 of TSHR [74].

The recently discovered role of TSHR gene variations in GD constitute a test case to understand the genetics of endocrine autoimmunity. We should thus expect that, following these epigenetic mechanisms just unveiled, we will gain a better understanding of the genetics of the whole group in the near future.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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