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THE CD40, CTLA-4, THYROGLOBULIN, TSH RECEPTOR, AND PTPN22 GENE QUINTET AND ITS CONTRIBUTION TO THYROID AUTOIMMUNITY: BACK TO THE FUTURE

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Abstract

Autoimmune thyroid diseases (AITD) are common autoimmune diseases affecting up to 5% of the general population. Thyroid-directed autoimmunity is manifested in two classical autoimmune conditions, Hashimoto's thyroiditis, resulting in hypothyroidism and Graves' disease resulting in hyperthyroidism. Autoimmune thyroid diseases arise due to an interplay between environmental and genetic factors. In the past decade significant progress has been made in our understanding of the genetic contribution to the etiology of AITD. Indeed, several AITD susceptibility genes have been identified. Some of these susceptibility genes are specific to either Graves' disease or Hashimoto's thyroiditis, while others confer susceptibility to both conditions. Both immunoregulatory genes and thyroid specific genes contribute to the pathogenesis of AITD. The time is now ripe to examine the mechanistic basis for the contribution of genetic factors to etiology of disease. In this review, we will focus on the contribution of non-MHC II genes to the etiology of AITD.

Keywords

Immunoregulation; Organ specific autoimmunity; Genetics; Autoimmune thyroiditis

INTRODUCTION

The thyroid, a major endocrine gland controlling diverse metabolic pathways is frequently affected by disease. Up to 5% of the overall population suffers from some form of autoimmune thyroid disease (AITD) [1-2], making AITD among the commonest autoimmune conditions. The current pathoetiological dogma for AITD is that it is a polygenic disease in which susceptibility genes and environmental triggers act in concert to initiate both cellular and humoral immune responses against the thyroid gland (reviewed in [3]). The exact nature of the environmental component to AITD is still not clearly understood; however, it is postulated to encompass factors such as dietary iodide, medications, and infection (reviewed in [4]). On the other hand, the genetic basis for AITD is becoming firmly established and represents one of the most exciting recent developments in the field of thyroid autoimmunity. There is a wealth of literature documenting a genetic contribution to the etiology of Graves' disease (GD) and Hashimoto's thyroiditis (HT) (reviewed in [3][5][6]). Both common and unique susceptibility

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genes exist for GD and HT [7]. The existence of shared susceptibility genes may be postulated to be based in the common origins of GD and HT, both of which are characterized by lymphocytic infiltrates reactive against thyroid antigens, and the production of thyroid-specific autoantibodies. Genes specific to either HT or GD could explain the different pathways that the two diseases take: the hallmark of GD is the production of TSH receptor stimulating antibodies causing hyperthyroidism, whereas HT is characterized by thyrocyte apoptosis leading to glandular destruction and ultimately clinical hypothyroidism (reviewed in [3][7])

Understanding AITD, at a molecular and genetic levels, promises to herald novel treatments and preventative modalities. This review will focus on non-MHC II genes that contribute to the etiology of AITD. The role of MHC genes and genetic regions linked with AITD in which no candidate gene has yet been identified have been reviewed elsewhere [3] and will not be discussed here.

THE GENETIC COMPONENT TO AITD

There was ample evidence to suggest that genetics are a major component in the etiology of AITD. Epidemiological data consistently pointed to a strong genetic predisposition to AITD (reviewed in [3]), and in particular, the familial occurrence of AITD had been reported by researchers for many years. Forty years ago, it was reported that the siblings of those affected by either GD or HT have a 33% chance of going on to develop AITD themselves [8]. Confirming these early observations, a recent analysis computed the risk of developing disease in siblings of patients, relative to the risk for the overall population, and thus generated a very high sibling risk ratios (λ_s) of 16.9 [9]. Additionally, thyroid antibodies (TAB's), which often portend the development of AITD, are found in ~50% of siblings of AITD patients [10][11][12][13][8], compared to a prevalence in the general population of 7-20% [1]. Finally, the strongest epidemiological evidence for a genetic susceptibility to AITD came from twin studies which have consistently shown significantly higher concordance rates for AITD in monozygotic twins than in dizygotic twins [14][15][16][17]; for Graves' disease, the concordance rate was 35% for monozygotic twins vs. 3% for dizygotic twins [14], and in Hashimoto's thyroiditis, the concordance rate for monozygotic twins was 38% vs. 0% for dizygotic twins [15].

Today, we have direct evidence for AITD susceptibility genes. Direct evidence for genetic contribution to AITD has come from whole genome screens, which have been followed up by subsequent linkage, association, and functional studies. In essence, this methodology provided an unbiased approach, as the entire human genome was scanned for linkage with disease, with no prior assumptions on gene function. Genome screens, as learned from initial studies with type 1 diabetes (T1D), identify major genes in disease pathology since they are unlikely to provide significant linkage for minor loci which only enhance the risk for disease [18][19][20][21][22][23]. Our group was the first to use this methodology to find AITD susceptibility genes [24]. The availability of large families with more than one affected individual, coupled with the mapping of highly polymorphic, closely-spaced markers covering the whole genome, has enabled the screening of the genome to identify AITD susceptibility genes (reviewed in [3]). Furthermore, a number of association studies have been conducted, looking for the presence of polymorphisms in candidate genes. Taken together, whole genome screens, along with candidate gene-based association studies, have yielded invaluable information towards understanding AITD, at a molecular level (reviewed in [3]). We summarize here the susceptibility genes that have emerged from these studies, and discuss the mechanistic bases for their contribution to thyroid autoimmunity.

THE *CD40* GENE AND GRAVES' DISEASE

CD40, a major B-cell and antigen presenting cell regulatory molecule

Of the Graves' disease susceptibility genes identified so far *CD40* is the only one regulating B-cell responses. A 45-50 kDa glycoprotein that is a member of the TNF-R receptor (TNFR) family of molecules [25], *CD40* was first identified and functionally characterized as a B lymphocyte activation molecule [26] [27][28], as it is expressed on non-terminally differentiated B cells during all stages of development [27][29][30]. Indeed, *CD40* plays a fundamental role in B-cell potentiation. The ligation of *CD40* affords the requisite co-stimulatory signal for: B-cell proliferation [31][27][32][33][34][35][36][37], immunoglobulin class switching [38], antibody secretion [39][40][41], the prevention of apoptosis of germinal-center B-cells [42], affinity maturation, and the generation of long-lived memory cells [43]. B cells are stimulated through the engagement of *CD40*, by its ligand *CD154*, which is presented by activated *CD4+* T helper cells [33][32]. The B cell – T cell interaction is a tightly controlled event that takes place at the interface of the parafollicular cortex and the lymphoid follicle in peripheral lymphoid tissues [44][45]. A *CD40* stimulated B cell can enter germinal centers and mature into a B cell clone that secretes large titers of high affinity antibodies [46][47][48] [49]. A stimulated B cell, receiving no secondary *CD40* signal, will have a limited lifespan and will make modest amounts of low-affinity antibodies, restricted to the IgM subtype. The importance of *CD40* to B cell activation and the development of a potent humoral response has been underscored by the profound immune system defects seen both in *HIGM3* (hyper IgM) patients [50], harboring mutations in *CD40*, and in *CD40* gene targeting experiments in mice [51][52].

Notwithstanding its place as a cardinal B cell regulatory receptor, more recently, it has become clear that *CD40* has a more general role and enjoys a promiscuous expression pattern and functionality that takes it beyond its original niche, as a B-cell activation molecule. Professional antigen presenting cells such as macrophages [53] and dendritic cells [54] too require *CD40* signaling for their activation and, furthermore, utilize *CD40* as a co-chaperone-like receptor, mediating the uptake of exogenous hsp70-peptide complexes [55]. Numerous studies have shown that *CD40* is widely expressed and functional on different cell types, including: endothelial cells [56][57][58], epithelial cells [59][60] [61], neuronal cells [62], hepatocytes [63], smooth muscle cells [64], fibroblasts [65], bone-marrow derived [54] and follicular dendritic cells [66], some carcinomas [67][29][68][69], monocytes [53], and even activated T cells [70]. Current research has demonstrated a role, for *CD40* in inflammation, proliferation, and apoptosis, as well [62][71][72][73]. Moreover, recent research has shown that *CD40*, long thought to be stationed at the cell surface, is capable of shuttling to the nucleus, where it can affect gene regulation [74].

CD40 and the link to disease

CD40 signaling cascade has been shown to play a role in a number of autoimmune conditions. For example, the introduction of antibodies against *CD154* blocked the development of both primary and secondary antibody responses [75], as well as ameliorated the effects of experimental autoimmune diseases, with a strong humoral component, such as collagen induced arthritis [76], lupus nephritis [77], experimental autoimmune myasthenia gravis [78], mercury-induced autoimmunity [79] and experimental Graves' disease [80]. Additionally, experiments conducted with Graves' patients thyroid tissue xenografted into severe combined immunodeficient (SCID) mice showed that the humoral response from these xenografted thyroids can be significantly inhibited when the *CD40/CD40* ligand interaction is disrupted [81].

Using whole genome scanning, we have identified *CD40* as a susceptibility gene for GD. Interestingly, *CD40* was associated with GD, but not with HT. Linkage studies, that we [82] [83] and others [84], performed, showed that the *CD40* gene locus was linked and associated with Graves' disease. Sequencing of the entire *CD40* gene led to the identification of a C/T polymorphism, strategically located in the Kozak sequence, a stretch of nucleotides flanking the start ATG codon in vertebrate genes, that is essential to the start of translation [85]. Case-control association studies demonstrated an association of the CC genotype with GD [83]. With the exception of a sole report [86], the association between the CC genotype and GD has now been replicated in several studies, performed in different populations including Caucasians [83][87], Koreans [88], and Japanese [89][90]. Additionally, a paper initially purporting a lack of association between the C allele and GD [91], was found, upon re-analysis, to actually show an association between the CC genotype and GD [92]. Moreover, a meta-analysis, encompassing all of the reported studies, showed a highly significant association between the CC genotype and GD [87].

How does the *CD40* Kozak SNP contribute to disease etiology?

Using a combination of *in vivo* and *in vitro* approaches, we demonstrated that the *CD40* Kozak SNP has important consequences in terms of *CD40* expression. The C allele of the polymorphism, whether in the context of a plasmid transfected into a cell line, or on the surface of a human B cell, increases the translational efficiency of nascent *CD40* mRNA transcripts, resulting in 15-32 % more *CD40* protein than that seen in the presence of the T allele [93]. We proposed that there exists a translational pathophysiological aspect to Graves' disease, due to subtle changes in the levels of *CD40* protein, with increased *CD40* expression contributing to disease etiology. This proposal has a paradigm, as there are a number of diseases whose etiology lies in altered efficiency of translation of a single, discrete gene. To date, several diseases, have been found to be caused by single nucleotide polymorphisms in the Kozak sequences of key genes (summarized in Jacobson *et al.* [93]).

At the level of the B cell, even modest changes in *CD40* expression levels could have profound consequences, and indeed we have demonstrated that the C allele increases the amount of *CD40* on the surface of B cells. As Grave's disease is a classical antibody-mediated disease, with antibodies against the thyroid stimulating hormone receptor, driving the proliferation of and concomitant excess thyroid hormone secretion by thyrocytes, B cells would be expected to play a major role in disease etiology. A B cell expressing a higher level of surface *CD40* may be expected to have a lower threshold for activation. Indeed, there are reports documenting that even a modest change in the expression levels of B cell surface receptors can precipitate an autoimmune condition [95]. There exist two general scenarios that can lead to the preponderance of peripheral, autoreactive B cells: alterations in B cell longevity, or alterations in cellular activation threshold [94]. A direct link between the overstimulation of B cells and autoimmune disease has been established. Transgenic mouse experiments have demonstrated that even a modest 15-29% increase in the expression levels of B cell molecule, *CD19*, is enough to initiate the development of systemic lupus erythematosus (SLE)-like disease in mice [95]. Complementing the results from the murine system was the finding that systemic sclerosis patients, on average, express *CD19*, on their B cells, at levels 20% higher with respect to healthy controls [95]. Like *CD19*, over-stimulation of the *CD40* pathway, too, has been linked with the elicitation of an autoimmune response. For example, previous reports have shown that overstimulation of murine B cells, by ectopically expressing a *CD154* transgene, leads to experimental SLE [96]. Additionally, a polymorphism in the 3'UTR of *hCD154*, that is associated with systemic lupus erythematosus, serves to cause a more prolonged protein expression in activated lymphocytes of patients versus those in controls [97]. Moreover, it has been demonstrated that B-cell activation via a *CD40* pathway leads to the over-production of IL-10 and a shift of the Th1/Th2 balance to Th2 dominance [98]. In terms of contribution to

Graves' disease etiology, CD40 could conceivably represent a trigger to autoimmunity in an autoreactive B-cell. An autoreactive B cell in the periphery, expressing constitutively higher levels of CD40, would have a lower threshold for stimulation and would be more easily activated. Hence, the C Kozak polymorphism of CD40 could increase the risk for developing humoral autoimmunity.

We would expect, but, as of yet have not proven, that the C Kozak allele would also enhance the efficiency of translation in other CD40-expressing tissues. Of most notable, would be the thyroid gland itself. It has been demonstrated that the CD40 is expressed and functional on thyrocytes [261] and the thyroidal expression of CD40 is upregulated in the context of Graves' disease [262]. Additionally, it is also known that under certain circumstances the thyrocyte can express MHC class II molecules and can act as a facultative APC [263][264]. Taken together, it could be postulated that there exist two, non-mutually exclusive mechanistic schemes that may help initiate or potentiate GD, by an over-expression of CD40 on thyrocytes, an intrinsic mechanism, and an extrinsic mechanism. The intrinsic mechanism would have a CD4+ T cell activate the CD40 signaling pathway in the thyrocyte, resulting in over-expression of certain cytokines such as IL-6 that could promote thyroid inflammation and autoimmunity by a bystander mechanism. Thyrocytes expressing more CD40, as in the context of the C allele, would be more readily affected. The extrinsic mechanism, on the other hand, proposes an enhanced co-stimulation of T cells by thyrocytes over-expression CD40. The CD4+ T cell would be polarized towards a Th2 response and would secrete cytokines that would serve to activate B cells; again B cells expressing more CD40 would more readily transverse the activation pathway, and thyrocytes expressing more CD40 would more readily activate CD4 + T cells.

Might the CD40 Kozak SNP play a role in other autoimmune conditions? This question remains open. The broad functionality of CD40, coupled with its importance in multiple experimental autoimmune conditions, suggest that the CD40 Kozak SNP could be associated with other autoimmune diseases in addition to Graves' disease. However, work from our group [83] and others [128] has shown that the CD40 Kozak polymorphism is not associated with either Hashimoto's thyroiditis or multiple sclerosis, both autoimmune disease that are Th1 in character. Interestingly, we also did not find an association of the CD40 Kozak SNP with Myasthenia Gravis, a classic antibody mediated autoimmune disease, similar to Graves' disease [unpublished results]. Thus, the CD40 Kozak SNP may be specific for GD. The basis for this specificity for GD is unknown, and awaits elucidation through additional studies.

THE *CTLA-4* GENE AND AUTOMMUNITY

CTLA-4: an attenuator of T cell activity

The cytotoxic T lymphocyte-associated factor 4 (CTLA-4) gene [99] was originally isolated from a cDNA library constructed by subtractive hybridization between cDNA from a cytotoxic T cell and mRNA from a B cell lymphoma clone [100], with the aim of finding a T cell activating gene. Notwithstanding, a combination of subsequent biochemical and mouse genetic studies have shown that CTLA-4, a 188 amino acid glycoprotein [99](reviewed in [101]), is in fact, a major negative regulator of T cell-mediated immune functions. For example, complexing CTLA-4 with a blocking monoclonal antibody was shown to orchestrate the proliferation of T cells and the production of IL-2 [102][103][104]. Additionally, CTLA-4 knockout mice succumb early in life due to aberrant activation T cell lymphocytes which infiltrate and destroy multiple tissues[105][106][107]; suggesting that, without CTLA-4, T cells can no longer exist in homeostasis.

We now know that CTLA-4 is not constitutively expressed on resting, naïve CD4+ CD25- T cells [108](and reviewed in [109]). Rather, in response to T cell receptor ligation, the expression

of CTLA-4 is induced, peaking 24-48 hours later (reviewed in [101]); [108][110][111]. Interestingly, even though CTLA-4 is a type I transmembrane protein of the immunoglobulin super family [99], it is estimated that, at most, only about 10% [111][112], of the total cellular CTLA-4, is expressed on the surface of T cells following activation of the TCR. CD4+CD25 + T regulatory cells constitutively express CTLA-4, although the requirement or lack thereof for CTLA-4 for their function is unclear (reviewed in [109]).

The CTLA-4 molecule initiates its signal in response to its ligation with either the B7-1 or B7-2 [113][114] proteins. In terms of connection to disease, CTLA-4 has been shown to be germane to a number of experimentally induced autoimmune conditions (reviewed in [115]). The administration of a soluble CTLA-4-Ig fusion protein has been shown, through competition with B7 ligands for binding CD28, to suppress murine lupus [116], collagen-induced arthritis [117], experimental autoimmune glomerulonephritis [118], and diabetes in NOD mice [119][120].

Polymorphisms and their effects on *CTLA-4* functionality

The CTLA-4 gene is a highly polymorphic gene. Several CTLA-4 polymorphisms have been found to be associated with autoimmunity, most notably a SNP at position +49 which results in an Ala>Thr substitution in the signal peptide, a 3' UTR AT dinucleotide repeat (microsatellite), and a SNP downstream the 3'UTR designated CT60 [121, see below]. Mechanistically, a polymorphism that compromises CTLA-4 functionality or reduces its cell surface expression would be expected to cause heightened T-cell activation, and potentially, lead to the development of an autoimmune condition. Several CTLA-4 SNPs and the 3'UTR microsatellite have been analyzed in detail for their effect on CTLA-4 function and/or expression. The A/G₄₉ SNP causing a Thr>Ala substitution in the signal peptide, was reported to cause mis-processing of CTLA-4 in the ER resulting in less efficient glycosylation and diminished surface expression of CTLA-4 protein [122]. However, no further studies have been performed on the effects of this SNP on post-translational modification of CTLA-4. Other workers have shown association between the G allele and reduced control of T cell proliferation [123][124]. However, this association could be due to a direct effect of the A/G₄₉ SNP or due to another polymorphism in linkage disequilibrium with the A/G₄₉ SNP. In order to examine whether the effect on T-cell proliferation was due to the A/G₄₉ SNP, Xu *et al.* [125] performed direct functional studies. They transiently transfected a T-cell line, devoid of endogenous CTLA-4 (Jurkat cells), with a CTLA-4 construct harboring either the G or the A allele of the A/G₄₉ SNP. They reported no difference in CTLA-4 expression and/or function when they transfected the cells with a CTLA-4 construct harboring the A or the G allele. Therefore, it was concluded that the A/G₄₉ is not the causative SNP. Two of the promoter SNPs have been studied for their effect on function, too. The -1661 SNP appears to be neutral, as there is no apparent change in promoter activity when either the A or G allele is present [126]. On the other hand, analysis of the -318 SNP, has revealed that T allele, in comparison to the C allele has an 18% higher promoter activity, as assessed in a luciferase based assay [127]. The results of this initial report have been subsequently corroborated, in another study [126]. Additionally, individuals carrying the T allele of the -318 polymorphism have been shown to have significantly elevated expression of CTLA-4 on the surface of stimulated cells, and significantly increased *CTLA-4* mRNA in resting cells [129]. Mechanistically, the -318 SNP may affect CTLA-4 levels by altering the binding of a transcription factor, LEF-1, whose binding site TT(C/T)AAG, encompasses the C/T polymorphism [126]. Studies analyzing the functional effects of the 3'UTR microsatellite have demonstrated that the longer repeats are associated with reduced CTLA-4 inhibitory function [130]. It is currently not known how the AT repeats affect CTLA-4 functionality or levels of available CTLA-4 protein. However, it should be noted that the region of CTLA-4, in which the AT repeats lie, the 3'UTR, is a strategic location, as there exist three AUUUA motifs which may affect mRNA stability [131][132]. Interestingly, we found an

association ([166] and unpublished results from our laboratory) between the protective allele (A) of the A/G₄₉ SNP, which is associated with increased CTLA-4 function/expression, and interferon induced thyroiditis (IIT) [133]. These preliminary data may suggest that CTLA-4 may contribute to autoimmunity by several mechanisms. Hence, we postulated that in the case of IIT, which develops in patients with hepatitis C infection [133], alleles associated higher levels of CTLA-4, could suppress T-cell function and may lead to a more severe infection and/or sequestration of the hepatitis C virus in the thyroid gland triggering thyroiditis. However, under most cases of spontaneous autoimmunity, alleles associated with reduced CTLA-4 function and/or expression could lead to increased activation of T cells thereby triggering autoimmunity.

CTLA-4 is associated with several autoimmune conditions

The CTLA-4 gene is located on chromosome 2q33 [134]. Considering its role as a negative regulator of T-cell activation, it comes as no great surprise that the CTLA-4 gene was found to be associated with a variety of autoimmune conditions [135]. The CTLA-4 gene locus was reported to be linked and/or associated with type 1 diabetes mellitus (T1D) [136][137][138], asthma [139], Addison's disease [140], myasthenia gravis [141], Sjorgren's syndrome [142] [143], systemic lupus erythematosus (SLE) [144] systemic sclerosis [145], ulcerative colitis [146] and with all forms of AITD (GD, HT, and TAb's [124], see below).

CTLA-4 association with thyroid antibodies (TAbs)

A whole genome scan conducted by our group demonstrated evidence for linkage between the CTLA-4 gene region and the production of thyroid antibodies (TAbs) with a maximum LOD score (MLS) of 4.2 [147]. In accordance with our finding another group reported an association between the G allele of the CTLA-4 A/G₄₉ SNP and thyroid autoantibody diathesis [148]. Moreover, the G allele of the A/G₄₉ SNP was also found to be associated with higher levels of both thyroglobulin and thyroid peroxidase autoantibodies [150]. Since the development of thyroid antibodies is often a harbinger for the clinical stage of AITD [149] it is possible that CTLA-4 predisposes, non-specifically, to the development of thyroid autoimmunity. Additional genetic and/or environmental factors are necessary for the development of the specific GD/HT phenotypes [135].

CTLA-4 associations with Graves' disease and Hashimoto's Thyroiditis

There is now solid data demonstrating an association between the CTLA-4 gene and AITD [151][152][153][154][155]. Initially, an association between the 3'UTR microsatellite and Graves' disease (GD) was found, yielding a relative risk of 2.1 to 2.8 [151][154]. Later, associations between the G allele of the A/G₄₉SNP and AITD were reported with a relative risk of ~ 2.0 [153][156][157][158][159][160]. These associations have been consistent across populations of different ethnic backgrounds, such as Caucasians [151], Japanese [159][161], and Koreans [162]. Furthermore, the association of CTLA-4 and GD has also been confirmed in a family based study using TDT analysis [163]. In contrast, association studies using the C/T₋₃₁₈ SNP of CTLA-4 have been considerably more varied with some showing association [158], while others [164] did not find association. More recently Ueda *et al.* [121] identified a new SNP (designated CT60) downstream from the 3'UTR region of CTLA-4 that showed the strongest association with GD. Functional studies have suggested that the associated allele of CT60 might modulate the alternative splicing of CTLA-4. However, these data await confirmation.

Since CTLA-4 is a negative regulatory molecule of T cells, with the potential to elicit effects in many different tissues, it could be postulated to confer susceptibility to AITD and autoimmunity in general and not specifically to GD [135]. This prediction has been born out,

as CTLA-4 has been reported to be associated with Hashimoto's thyroiditis in various populations including Caucasians [154][157][140], and Japanese [165][161].

CTLA-4 association with severity of autoimmune thyroid disease

There are a number of studies which, taken together, suggest that CTLA-4 may influence the severity of the AITD phenotype. Heward *et al.* [163] reported that the CTLA-4 A/G₄₉ SNP G allele was associated with more severe thyrotoxicosis at diagnosis (as reflected by higher free T4 levels). Similar findings were reported by Park *et al.* [162] but not by Zaletel *et al.* [148]. It has also been reported that the frequency of the G allele and the GG genotype of the CTLA-4 A/G₄₉ SNP was significantly higher in GD patients who did not go into remission after five years on anti-thyroid medications [167].

CTLA-4 polymorphisms have also been tested for association with Graves' ophthalmopathy (GO). Most studies have been negative and did not show that CTLA-4 conferred a specific risk for GO beyond that conferred for GD [156][168]. However, several groups have reported an association between GO and CTLA-4 [162][169][160]. It is our hypothesis that the reported CTLA-4 association with GO reflects an association between CTLA-4 and more severe GD and is not representative of a specific association with the GO phenotype. Indeed, our recent segregation analysis showed no evidence for a genetic susceptibility specific to GO beyond the genetic susceptibility to GD in general [156].

THYROGLOBULIN: A THYROID SPECIFIC SUSCEPTIBILITY GENE

Thyroglobulin (Tg), a 660 kDa homodimeric protein, that runs at 19S in classical ultracentrifugation studies [170], is a very large molecule that accounts for approximately 75-80% of total thyroidal protein [171]. Thyroglobulin serves as a precursor and veritable storehouse for the thyroid hormones T3 and T4 [172]. The biological importance of Tg is highlighted by the finding that several naturally occurring mutations in Tg are associated with profound biological consequences (comprehensively reviewed in [173]). Interestingly, despite the fact that thyroglobulin is intimately associated with the thyroid, it is a normal component of the blood, as some thyroglobulin invariably leaks, during normal thyroid hormone secretion [174].

The thyroglobulin protein molecule undergoes several important post-translational modifications, including iodination [175], glycosylation [176][177], sulfonation [178], and phosphorylation [179][180][181]. The varying degree of the posttranslational modifications of thyroglobulin, particularly iodination [182], bestow a heterogeneous character upon the thyroglobulin molecule. In terms of relevancy to disease, some of these posttranslational changes have been proposed to influence the initiation of thyroid autoimmunity. Iodination, the most studied of these modifications, is believed to be a contributor to disease. The thyroglobulin protein molecule undergoes iodination by thyroid peroxidase (TPO) [183][184][185], and there are reports suggesting that iodination of thyroglobulin alters Tg immunoreactivity, and produces a higher grade thyroiditis in the EAT model (reviewed in [186]). However, reports showing that the immunogenicity of peptides, containing potential sites of iodination, is a function of amino acid sequence rather than iodination [187][188], coupled with the finding that T cells from the thyroiditis prone Buffalo rat, respond equally well to thyroglobulin prior to disease onset irrespective of its iodine content [189], may suggest that iodinated thyroglobulin is not a prerequisite for the initiation of thyroiditis, but may exert an effect on disease maintenance and/or severity. Another possibility is that the iodine itself, rather than iodinated thyroglobulin, may enhance thyroiditis by direct toxic effects on the thyroid. Indeed, iodine has been reported to cause necrosis both in human thyroid follicles [190] and in hyperplastic thyroid glands of normal animals [191][192], and to induce apoptosis in thyroid cells [193]. In addition, glycosylation, has also been demonstrated to be important

in thyroglobulin antigenicity [194]. Regardless of which posttranslational modification influences the initiation of thyroid autoimmunity, it can not be disputed that the molecule represents one of the most important targets in AITD.

Anti-Thyroglobulin autoantibodies in Hashimoto's thyroiditis

Hashimoto's thyroiditis (HT), the most frequent form of AITD has as its hallmark an intense thyroidal lymphocytic infiltrate which gradually destroys the gland, culminating in clinical hypothyroidism [195][196]. Thyroglobulin autoantibodies are prevalent in HT, with high titers of IgG anti-Tg autoantibodies being found in > 80% of HT patients [197]. Furthermore, 94% of Tg-antibody positive HT patients also have antibodies to TPO [198]. The humoral response to Tg in HT patients is specific, since it is characterized by the presence of B cells showing increasing degrees of somatic hypermutation as they produce antibodies with increasing affinities for Tg [199][200]. This is in contrast to Tg antibodies found in approximately 27% of normal, healthy individuals in the United States [201], which differ from those seen in HT patients, by being polyreactive [202][203], of lower affinity [204], and of predominantly IgM isotype [205].

Murine systems and the importance of Thyroglobulin

Mouse models have provided strong evidence for the importance of thyroglobulin in the development of thyroid autoimmunity. The currently accepted model for Hashimoto's thyroiditis, murine experimental autoimmune thyroiditis (EAT) can be induced, in genetically susceptible mice, by immunization with either autologous or heterologous thyroglobulin, in conjunction with complete Freund's adjuvant or with lipopolysaccharide (reviewed in [206]). EAT, like its human disease counterpart, is characterized by a cellular infiltrate of the thyroid [172], as well as high titers of anti-Tg autoantibodies [207] and *in vitro* splenocyte proliferation, in response to Tg [208]. CD4+ T cells have been shown to play a pivotal role in the induction [209][210][211][212], as well as in the suppression [213][214][215], of EAT in mice.

Whole genome scans reveal an autoimmune thyroid disease locus on chromosome 8q

Even though GD and HT have opposing clinical manifestations, there is evidence for a commonality in their pathogenesis [2][216]. Both conditions are marked by lymphocytic infiltration of the thyroid (although markedly more intense in HT), proliferative responses of T lymphocytes against thyroid autoantigens, and production of antibodies against thyroid antigens (reviewed in [217]). Indeed, our linkage studies have suggested that the common etiology of GD and HT is partly determined by genetic factors, as several loci have shown linkage to both GD & HT [7][218]. One locus on chromosome 8q24 was found to be strongly linked with AITD in two whole genome screens [7][219]. Fine mapping of this locus showed that the AITD susceptibility gene in this region was the Tg gene [220]. Furthermore, case control and family association studies, using microsatellites in introns 10 and 27 of the Tg gene demonstrated an association of the Tg gene with AITD [220][221][265]. Thus, we hypothesized that polymorphisms in the thyroglobulin gene may predispose to AITD. As such, subsequent to the identification of the thyroglobulin gene as an AITD susceptibility gene, we sought to determine whether there were any intragenic variations which influenced disease.

Thyroglobulin Missense SNPs and their interaction with MHC II molecules

Detailed sequence analysis of the entire *hTg* gene has revealed 4 intronic SNPs and 10 exonic SNPs. Subsequently, on the 14 SNPs, we performed case control association studies in a sample pool of 240 AITD patients and 150 healthy controls [222]. One SNP cluster, in strong linkage disequilibrium, (residing in exons 10-12), and a SNP in exon 33 showed significant associations with AITD. As further support for the contribution of thyroglobulin polymorphisms to disease, we identified missense SNPs in the thyroglobulin gene that were associated murine

autoimmune thyroiditis [222]. Further studies have shown that the *hTg* SNPs may interact with HLA-DR sequence variants in predisposing to GD, suggesting a biochemical interaction of thyroglobulin with the MHC II molecules.

The presentation of an antigenic peptide, bound by an MHC II molecule, on the surface of professional antigen presenting cells (APC's) (e.g., dendritic cells, macrophages, & B cells), to the T cell receptor on CD4+ T lymphocytes, represents a cornerstone in the initiation of an adaptive immune response [223]. The MHC II molecule exists as a heterodimeric complex, consisting of an alpha chain and a beta chain, which come together to form a cleft that can accommodate peptides of 10-30 residues [224][225]. Solid genetic evidence now exists showing an association of MHC class II molecules with autoimmune thyroid disease [226][227][228][229]. In addition to AITD, many other autoimmune diseases are associated with MHC II genes [230]. Hence, it is likely that MHC II peptide presentation plays a major role in the initiation of an autoimmune response. Indeed, structural studies in several MHC II-associated autoimmune diseases have shown that susceptibility to disease is caused by certain structural features of the peptide binding cleft, of the relevant MHC II protein, that determine their interaction with immunogenic peptides [224][231][232][233][234].

Work from our laboratory has demonstrated that a single amino acid variation in the peptide binding cleft of HLA-DR, resulting in an arginine at position 74 of the beta chain, was strongly associated with Graves' disease (GD), while the presence of a glutamine at the same location was protective [235]. Another large study, from the UK, corroborated the association of Arg74 with GD [236]. Further analysis showed that the SNP in exon 33 of *Tg*, had a statistical interaction with the Arg74 polymorphism of HLA-DR, resulting in a high odds ratio for GD. This statistical interaction may imply a biological interaction between *Tg* and HLA-DR. How can *Tg* SNPs and HLA-DR-Arg74 interact biologically to increase the risk for GD? One putative model is that the *Tg* peptide repertoire that is generated due to the associated *Tg* SNP alleles is pathogenic, while DR β -Arg74 is able to more optimally present these pathogenic *Tg* peptides to T-cells. Thus, inheriting both the disease-associated *Tg* SNP alleles and DR β -Arg74 would result in the production of pathogenic *Tg* peptides and in their efficient presentation to T-cells (Figure 1).

THE *TSH RECEPTOR (TSHR)* GENE AND GRAVES' DISEASE

The thyroid hormone receptor, expressed on the surface of thyroid epithelial cells, binds thyroid stimulating hormone (TSH) and through the consequent activation of an adenylate cyclase and phosphatidylinositol-mediated pathway, signals for the production of thyroid hormones [237]. The hallmark of Graves' disease is the presence of stimulating TSHR autoantibodies. Not surprisingly, the TSHR gene was long thought to be an obvious candidate gene for GD. To date, three common germline SNP's of the TSHR have been described [238]. Two of these SNPs reside in the extracellular domain of the TSHR; they are: an aspartic acid to histidine substitution at position 36 (D36H), and a proline to threonine substitution at position 52 (P52T). The third SNP, a relatively conservative substitution of glutamic acid for aspartic acid (D727E), lies within the intracellular domain of the receptor. Bahn and colleagues were the first to report an association between the P52T SNP of the TSHR extracellular domain and GD [239]. However, another large study reported no association of the TSHR with GD [240], and therefore it was unclear whether the TSHR is indeed an important susceptibility gene for GD. Further, studies gave inconsistent results [241][242][243][244]. However, one group in Japan consistently reported associations of the TSHR with GD in the Japanese [161][165][245]. More recently, a very extensive recent study which tested a large number of SNP spanning the entire TSHR gene demonstrated a significant association of SNPs in intron 1 of the TSHR with GD [246]. This association was found in two very large independent Caucasian datasets thereby

confirming the association of TSHR variants with GD [246]. However, the magnitude of the contribution of the TSHR to GD susceptibility remains to be determined.

THE PROTEIN TYROSINE PHOSPHATASE-22 (PTPN22) GENE

The lymphoid tyrosine phosphatase (LYP), encoded by the *protein tyrosine phosphatase-22* (PTPN22) gene, embodies a 110 kDa protein tyrosine phosphatase [247] that, like CTLA-4, is a powerful inhibitor of T cell activation [248][249]. Recently, a tryptophan for arginine substitution at codon 620 (R620W) of the LYP protein was found to be associated with rheumatoid arthritis [250] and SLE [251]. Further studies showed that the PTPN22 R620W SNP was associated with other autoimmune diseases including type 1 diabetes mellitus (T1D) [252][253], Graves' disease [254], and Hashimoto's thyroiditis [255]. This SNP was found to elicit a functional change in LYP, such that the tryptophan-bearing LYP allele cannot associate with C-terminal src kinase (Csk), a partner molecule in an inhibitory complex that regulates key T cell receptor signaling kinases (Lck, Fyn, ZAP-70) [252]. Somewhat paradoxically, this mutation makes the protein an even stronger inhibitor of T cells, as it is a gain-of-function mutation [256]. It is speculated that a lower T cell signaling would lead to a tendency for self-reactive T cells to escape thymic deletion and thus remain in the periphery. Studies in different geographic regions revealed ethnic differences in associations most probably due to founder effects and/or due to the or absence of certain variants in certain ethnic groups. As an example the tryptophan variant of the protein tyrosine phosphatase-22 (PTPN22) gene is not found in the Japanese and, therefore, this gene does not contribute to autoimmunity in the Japanese [257].

BACK TO THE FUTURE

It is likely that in the near future novel susceptibility genes for AITD will be identified, and the mechanisms through which they cause disease will be unravelled. The linkage and association studies of tomorrow will be performed with a fraction of the speed with which today's screens are being done. A major contributor to the advance and increase in efficiency of genetic studies is the HapMap project [258]. To date, the HapMap has characterized haplotype structures across the genome of four human populations. This project which has divided the genome into linkage disequilibrium blocks enabled the utilization of "tagged" SNPs (representing linkage disequilibrium blocks) to test large genomic areas (or the entire human genome) for association with disease. Moreover, the HapMap coupled with microarray-based genotyping technology, enable the typing of up to 500,000 SNPs in a single experiment. Indeed, whole genome association studies have already proven useful in identification of new autoimmune disease genes [259].

We believe that the genome-wide association studies of the future, coupled with the findings from previous linkage and association studies, will brandish the susceptibility genes that predispose to thyroid autoimmunity. The identification of novel AITD genes and the understanding of the mechanisms by which they cause disease will enable, in the future, rapid identification of those individuals at risk for developing AITD before the appearance of overt symptoms. Treatment will, thus, evolve towards more preventive strategies, as opposed to the palliative methods of the present. For those individuals who do go on to develop clinical disease, their treatment will be personalized by targeting the specific pathways that are most contributory to an individual's subtype of AITD. We believe that the future will witness the engagement and deployment of novel molecules, such as altered peptide ligands [260] and specific monoclonal antibodies, as part of an arsenal designed to modulate specific autoimmune pathways in affected individuals, thus treating the cause of the disease and not simply suppressing the immune response.

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1. Ribbon diagram of the HLA-DR peptide-binding pocket showing a putative immunogenic thyroglobulin (Tg) peptide that binds with high affinity to a pocket containing the amino acid arginine at position 74 of the DR beta chain. Such a (Tg) peptide could be efficiently presented to T cells resulting in a strong immune response to thyroglobulin.