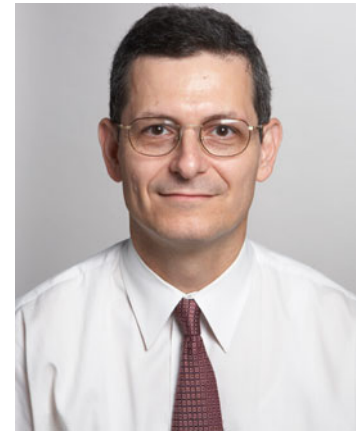


Genetic and epigenetic mechanisms in thyroid autoimmunity

Alia Hasham · Yaron Tomer



Yaron Tomer

© Springer Science+Business Media, LLC 2012

Abstract Autoimmune thyroid diseases (AITD), including Graves' disease and Hashimoto's thyroiditis, are among the commonest autoimmune disorders, affecting approximately 5 % of the population. Epidemiological data support strong genetic influences on the development of AITD. Since the identification of HLA-DR3 as a major AITD susceptibility gene, there have been significant advances made in our understanding of the genetic mechanisms leading to AITD. We have shown that an amino acid substitution of alanine or glutamine with arginine at position 74 in the HLA-DR peptide binding pocket is a critical factor in the development of AITD, and we are continuing to dissect these mechanisms at the molecular level. In addition to the MHC class II genes, there are now several other confirmed gene loci associated with AITD, including immune-regulatory (CD40, CTLA-4, PTPN22, FOXP3, and CD25) and thyroid-specific genes (thyroglobulin and TSHR). Mechanistically, it is postulated that susceptibility genes interact with certain environmental triggers to induce AITD through epigenetic effects. In this review, we summarize some of the recent advances made in our laboratory dissecting the genetic–epigenetic interactions underlying AITD. As shown in our recent studies, epigenetic modifications offer an attractive mechanistic possibility that can provide further insight into the etiology of AITD.

Keywords Thyroid genetics · Autoimmune thyroid disorders · Epigenetics

Introduction

Autoimmune thyroid diseases (AITD), comprised of Graves' disease (GD) and Hashimoto's thyroiditis (HT), are reported to be the commonest autoimmune endocrine disorders, affecting up to 5 % of the population. Although both conditions are characterized by infiltration of the thyroid by T and B cells reactive to thyroid antigens with the production of thyroid-specific antibodies, the result is two clinically opposing syndromes. In GD, lymphocytic infiltration leads to the activation of TSH receptor (TSHR)-

reactive B cells that produce TSHR-stimulating antibodies, manifesting as hyperthyroidism. In contrast, HT is characterized by apoptosis of thyroid cells, resulting in clinical hypothyroidism.

Considerable progress has been made to further our understanding of the mechanisms leading to thyroid autoimmunity. Although the exact etiology is not yet known, AITD is thought to arise from an interaction between genetic susceptibility factors, epigenetic effects, and various environmental triggers (e.g., iodine, infection), which leads to breakdown of tolerance. While the environmental component is still to be clarified, the genetic contributions to AITD are gradually being deciphered at a molecular level and are now more clearly understood. To date, there are at least seven genes that have been shown to contribute to the etiology of AITD. There is now evidence that each gene alone exerts weak effects, but together these genes interact to produce stronger, combined effects, ultimately causing disease. With recent advances in genomic

A. Hasham · Y. Tomer (✉)
Division of Endocrinology, Department of Medicine, Mount Sinai Medical Center, One Gustave L. Levy Place, Box 1055, New York, NY 10029, USA
e-mail: Yaron.Tomer@mssm.edu

A. Hasham · Y. Tomer
James J. Peters VA Medical Center, Bronx, NY 10468, USA

sequencing and analysis tools, and the completion of the HapMap project and the 1,000 genome project, these developments will help provide greater insight into the pathogenesis of thyroid autoimmunity. This review summarizes the more recent, major advances in our understanding of the etiology of AITD, with focus on our laboratory's work on the contribution of the HLA-DR, CD40, and thyroglobulin (Tg) genes.

Brief summary on environmental factors and AITD

There have been numerous environmental triggers identified as risk factors for the development of AITD. These include iodine (excess or deficiency), medications (e.g., interferon-alpha (IFN α) and amiodarone), bacterial and viral infections (e.g., *Yersinia Enterocolitica* and hepatitis C virus), smoking, stress, and pollutants (e.g., polyaromatic hydrocarbons) (reviewed in [1]). Potential mechanisms by which these agents induce disease may involve interference with thyroid function, direct toxic effects on thyroid cells, and/or immune stimulation [2]. For example, iodine, while essential for normal thyroid function, could induce autoimmunity in the thyroid by increasing immunogenicity of a highly iodinated thyroglobulin molecule or by releasing free oxygen radicals as a result of direct tissue toxicity [3]. Although initial data were conflicting, recent studies have substantiated hepatitis C virus (HCV) as an important infectious trigger of AITD. In addition, IFN α , a commonly used therapeutic agent in HCV infection, is recognized to be strongly associated with the development of thyroiditis [4]. The clinical manifestations of AITD are likely a result of complex interactions between environmental and genetic susceptibility factors. Although the exact nature of this interaction remains unclear, one possible mechanism is through epigenetic modifications.

Genetic contribution to AITD

Epidemiological studies have long recognized the important role of genetics in the etiology of AITD. Twin studies have estimated that 79 % of the attributed cause is due to genetic factors, with higher concordance rates for AITD observed in monozygotic twins compared to dizygotic twins (reviewed in [5, 6]). Other studies have reported that siblings of those affected by GD or HT have a 33 % chance of developing disease (reviewed in [7]). The first gene found to be associated with both GD and HT was HLA-DR3. Since this discovery, significant progress has been made in the genetic contributions and the mechanisms underlying thyroid autoimmunity. To date, several loci have been associated with AITD. In addition to HLA-DR

Table 1 Classification of AITD susceptibility genes

MHC Class II	Immune-regulatory	Thyroid-specific
HLA-DR	CD40	Thyroglobulin (Tg)
	CTLA-4	TSHR
	PTPN22	
	FOXP3	
	CD25	

subtypes, these include 2 groups of non-MHC genes: (1) immune-regulatory genes (CD40, CTLA-4, PTPN22, FOXP3, and CD25) and (2) thyroid-specific genes (Tg and TSHR) (Table 1).

HLA-DR gene locus and AITD

Genetic studies: the key role of HLA-DR3

The major histocompatibility complex (MHC) gene locus, located on chromosome 6p21, is a highly polymorphic genetic region and encodes the HLA glycoproteins. The HLA region contains several immune response genes found to be associated with various autoimmune disorders and is therefore the first candidate genetic region to be studied for association with AITD. The genes, encoded by the MHC locus, are divided into 3 distinct groups: (1) class I genes include the HLA antigens A, B, and C, (2) class II genes include heterodimeric HLA-DR, DP, and DQ genes, and (3) class III genes include complement components (e.g., C4), heat shock protein 70 (hsp70), tumor necrosis factor alpha, among others (reviewed in [5]).

Earlier studies analyzed the major HLA class II alleles and identified a significant association of GD with HLA-DR3 (HLA-DRB1*03) [8]. While 15–30 % of the general population have been found to possess the DR3 allele, the frequency in Caucasian individuals with GD is approximately 40–55 % (reviewed in [5]). Furthermore, we have recently shown that HLA-DR3 contributes significantly to the joint genetic susceptibility in families with both type 1 diabetes (T1D) and AITD [9, 10]. In contrast, earlier studies concerning an association between HLA-DR3 and HT have overall been less conclusive than that with GD, with several associated HLA-DR alleles being reported (reviewed in [11]). These discrepancies may also be attributed to differences in disease definition, as there is a wide spectrum of clinical manifestations seen in HT.

Mechanistic studies: HLA-DR and GD

The mechanisms via which HLA molecules induce autoimmunity are slowly beginning to be unraveled at a molecular level. Over 25 subtypes of HLA-DR3 are

Table 2 List of HT-associated amino acid residues in HLA-DR, their positions in peptide binding pockets, and changes upon amino acid substitutions

Residue	MHC Pocket	Susceptible	Resistant	Changes
β 26	P4 (bottom)	Tyr ($p = 6.9 \times 10^{-4}$; OR = 2.52)	Leu ($p = 0.02$; OR=0.50)	Smaller in size Loss of -OH
β 30	P6 (bottom)	Tyr ($p = 7.5 \times 10^{-4}$; OR = 2.11)	His ($p = 7.6 \times 10^{-4}$; OR = 0.07)	Smaller in size Positive, polar Loss of -OH
β 70	P4 (above)	Gln ($p = 4.3 \times 10^{-3}$; OR = 1.65)	Arg ($p = 0.03$; OR = 0.40)	Larger in size Positive
β 71	P4 (side)	Lys ($p = 1.7 \times 10^{-8}$; OR = 2.98)	Arg ($p = 7.2 \times 10^{-5}$; OR = 0.47)	Larger in size
β 74	P4 (side)	Arg ($p = 1.5 \times 10^{-4}$; OR = 3.40)	Gln/Leu ($p = 0.04$; OR = 0.63)	Smaller in size Uncharged

Reproduced with permission from the Proceedings of the National Academy of Sciences of the United States of America. Menconi et al. [16].
© The National Academy of Sciences

known, each with unique protein sequence. Recently, we have identified the exact amino acid sequence in the DR β 1 chain, which confers susceptibility to GD [12]. Since each of the HLA alleles is likely to have different binding affinities for peptides, we hypothesized that specific pocket amino acid sequences are capable of binding autoantigenic thyroidal peptides, (i.e., thyroglobulin (Tg), thyroid peroxidase, and thyrotropin receptor), ultimately resulting in thyroid autoimmunity. This hypothesis has been confirmed for several autoimmune disorders, most notably T1D, in which the amino acid residue at position 57 of the DQ β chain was found to play a key role in the genetic susceptibility to T1D [13, 14]. Similarly, focusing on the binding pocket of HLA-DR3, we have discovered a key mechanism that can provide insight into the etiology of AITD. We sequenced the HLA-DR β 1 locus in a group of Caucasian GD patients and in controls and subsequently identified substitution of the neutral amino acids alanine and glutamine for the positively charged arginine residue at position 74 of the HLA-DR β 1 chain (HLADR β 1-Arg74) [12]. This unique, three-dimensional, structural change in the HLA-DR peptide binding pocket, located in pocket 4 of the binding cleft, likely allows pathogenic peptides to bind to the HLA molecule and be recognized by autoreactive T cells, conferring susceptibility to GD. Conversely, the presence of glutamine at position 74 of this peptide binding pocket was determined to be protective for GD, collectively suggesting that position 74 is critical for the initiation of GD. These findings have also been confirmed by a large study performed in the UK [15].

Mechanistic studies: HLA-DR and HT

Similar to the HLA-DR pocket variant found in patients with GD, it was thought that there are specific sequence

variants within the peptide binding pocket that confer risk of HT. We sequenced the polymorphic exon 2 of the HLA-DR gene in a cohort of well-characterized patients with HT and compared them to HLA-DR sequences in matched controls [16]. The DR3 allele had a significantly higher frequency in patients than in controls ($p = 2 \times 10^{-3}$, odds ratio (OR) = 2.7). As previously shown in GD, the key pocket amino acid identified in HT was also DR β 1-Arg-74. More specifically, the amino acid sequence found to confer risk of HT was comprised of Tyr-26, Tyr-30, Gln-70, Lys-71, and Arg-74, with Lys-71 and Arg-74 conferring the greatest risk. On the other hand, the presence of HLA-DR amino acids Leu-26, His-30, Arg-70, Arg-71, and Gln-74 demonstrated protective effects [16] (Table 2). In addition, to determine further susceptibility variants, we sequenced exon 2 of the I-E gene in 22 strains of mice, 12 EAT-susceptible and 10 EAT-resistant. The I-E locus of the mouse and the human HLA-DR locus are homologous. Interestingly in mice, different pocket amino acids were identified to be strongly associated with EAT, namely Val-28, Phe-86, and Asn-88. These data suggest that different antigenic epitopes are involved in the development of AITD in mice compared to humans [16].

To provide insight into the functional consequences of these amino acid variants found to be associated with HT, we developed structural models of HT-susceptible and HT-resistant HLA-DR, based on genetic association studies. The electrostatic potential on the surface of both molecules was characterized in order to compare the two models. The major difference was in the P4 pocket, which caused a three-dimensional structural alteration that was a critical determinant for the development of HT [16] (Fig. 1). An explanation for this finding is that the P4 pocket in HT-susceptible HLA-DR (β 74-Arg) is more positively charged than in HT-resistant HLA-DR (β 74-Gln/Leu). This

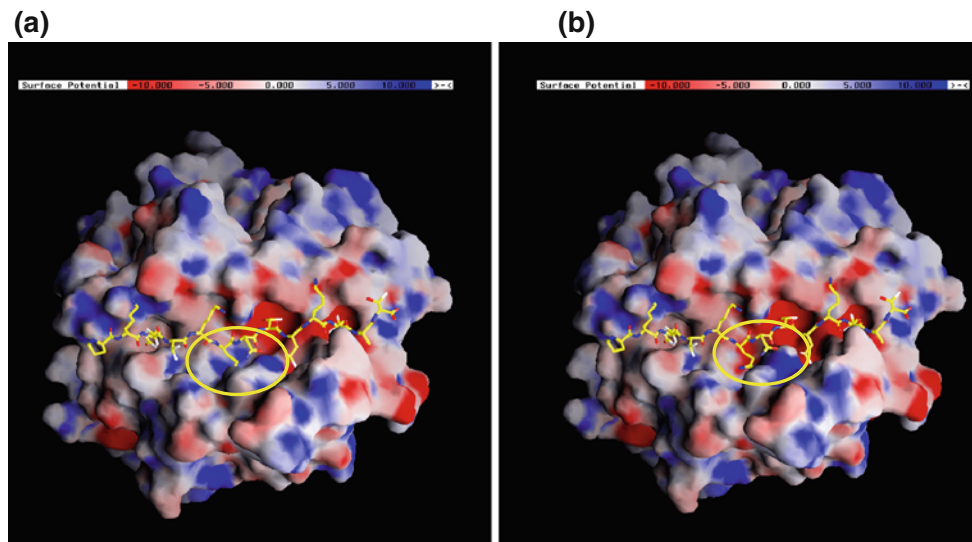


Fig. 1 Structural models of HT-susceptible and HT-resistant HLA-DR4. **a** HT-susceptible HLA-DR4. **b** HT-resistant HLA-DR4. The peptide binding groove is shown with the influenza hemagglutinin peptide. A significant difference was found in P4 pocket (yellow

ellipse), which in HT-susceptible HLA-DR4 is more positively charged (Reproduced with permission from the Proceedings of the National Academy of Sciences of the United States of America. Menconi et al. [16]. © The National Academy of Sciences) (Color figure online)

structural change can affect the selectivity and binding of pathogenic peptides (i.e., TPO or Tg-derived) to these disease-associated pockets, influencing the risk of HT.

HLA-DR and thyroid autoantigens

The amino acid variants (i.e., Arg74/Glu74) and the resulting structural changes in pocket 4, as described above, may enable binding of certain thyroid-derived, pathogenic peptides, such as from thyroglobulin (Tg) and TSHR, two major thyroid proteins that have also been shown to predispose to AITD. However, few studies have analyzed the mechanisms by which these thyroidal peptides are presented to T cells by the different HLA-DR variants. Indeed, we have identified an interaction between DR β 1-Arg74 and Tg-derived peptides that predisposed individuals to GD [17]. The importance of Tg as a target in thyroid autoimmunity is discussed in more detail below.

Thyroid-specific genes and AITD: Thyroglobulin

Genetic studies: the multidimensional role of thyroglobulin

Thyroglobulin (Tg) accounts for approximately 75–80 % of total thyroidal protein and serves as a precursor to thyroid hormones, T3 and T4. Due to the fact that AITD phenotypes, GD and HT, are characterized by the development of Tg antibodies, Tg has been recognized as a key antigen and obvious target gene in thyroid autoimmunity. The Tg molecule undergoes important post-translational modifications, including iodination, glycosylation, sulfonation, and

phosphorylation. These molecular changes are also thought to contribute to the development of thyroid autoimmunity.

We performed whole-genome linkage studies and mapped, in a data set of 102 multigenerational AITD families, 3 loci on chromosomes 6p, 8q, and 10q, providing evidence for linkage with the entire AITD data set [18]. Specifically, the 8q24 locus, which contains Tg, was shown to be strongly linked to AITD, findings that were observed in other ethnic groups [19]. Sequencing all 48 exons of the Tg gene subsequently identified 14 SNPs. Association studies performed on these SNPs revealed that an exon 10–12 SNP cluster and an exon 33 SNP were significantly associated with AITD [20]. Three of these SNPs were non-synonymous, resulting in amino acid sequence variations in Tg [20]. As described above, we have shown that arginine at position 74 of the HLA-DR β 1 chain confers susceptibility to GD. To investigate epistatic effects, we examined for gene–gene interaction between DR β 1-Arg74 and the associated Tg SNPs. Intriguingly, a significant interaction was found between HLA-DR β 1-Arg74 and the W1999R variant of Tg, yielding an odds ratio (OR) of 15.0 for GD [17, 20]. Logistic regression analysis showed that these two risk alleles act synergistically, resulting in a higher combined OR than would be produced from the additive effects of these gene variants alone [17]. The biological basis of this Tg:HLA-DR variant interaction could be explained by increased pathogenicity within the Tg peptide repertoire combined with an optimized ability of DR β 1-Arg74 to present these pathogenic Tg peptides to T cells. Therefore, when these two disease-associated alleles are inherited together, the risk of developing GD is substantially increased.

To better understand the features of the HLA-DR peptide binding pocket and its influence on Tg peptide binding, we designed a recombinant HLA-DR3 expression system to examine binding to select, cathepsin-generated Tg peptides [21]. Cathepsins B, D, and L, endosomal proteases, are involved in antigen processing, as well as in Tg degradation, thereby creating the Tg peptidic repertoire that is available to be presented to T cells. We tested the binding of cathepsin-generated Tg peptides to recombinant HLA-DR molecules that contained either AITD-susceptible or AITD-resistant DR pocket sequences [21] (Fig. 2). Using this approach, we were able to successfully identify 4 Tg peptides that bound strongly and specifically to the recombinant AITD-susceptible, HLA-DR3 molecule, Tg.726, Tg.1571, Tg.1951, and Tg.2098. We performed an analysis of the interaction energies between the specific residues of the Tg peptides and the MHC protein [21] (Fig. 3). Localized entirely to the HLA-DR β subunit, the strongest and most important pairwise interactions were noted on residues Lys- β 71 and Arg- β 74, suggesting that these sequence substitutions clearly impact peptide binding and selectivity [21].

Recently, we sequenced the promoter region of Tg up to 2.5 Kb upstream the start codon and identified a new variant that was associated with AITD. This variant is an A/G SNP located at position -1623 (rs180195). Association studies demonstrated a strong association with AITD in Caucasian AITD patients ($p = 0.006$) [22] (Fig. 4). Furthermore, transmission disequilibrium test (TDT) analysis in families with AITD confirmed the association ($p = 0.034$).

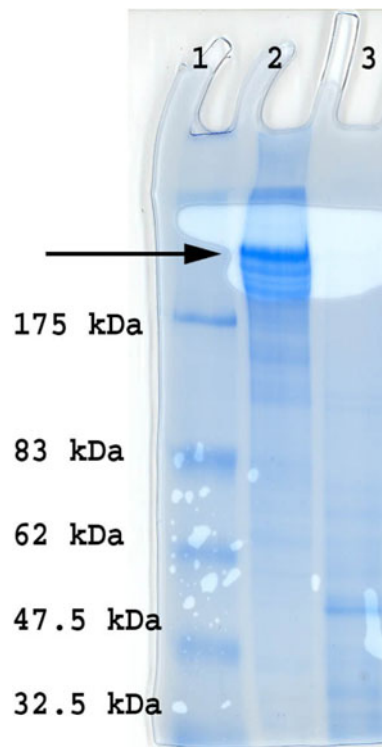


Fig. 2 Undigested and cathepsin-digested Tg run on a 7.5 % polyacrylamide gel. *Lane 1*, Molecular weight markers. *Lane 2*, Undigested thyroglobulin, under reducing conditions, showing a predominant 330-kDa band (arrow) that corresponds to a Tg monomer. *Lane 3*, thyroglobulin after treatment with cathepsin D (Reproduced with permission from the American Society for Biochemistry and Molecular Biology. This research was originally published in The Journal of Biological Chemistry. Jacobson et al. [21]. © The American Society for Biochemistry and Molecular Biology)

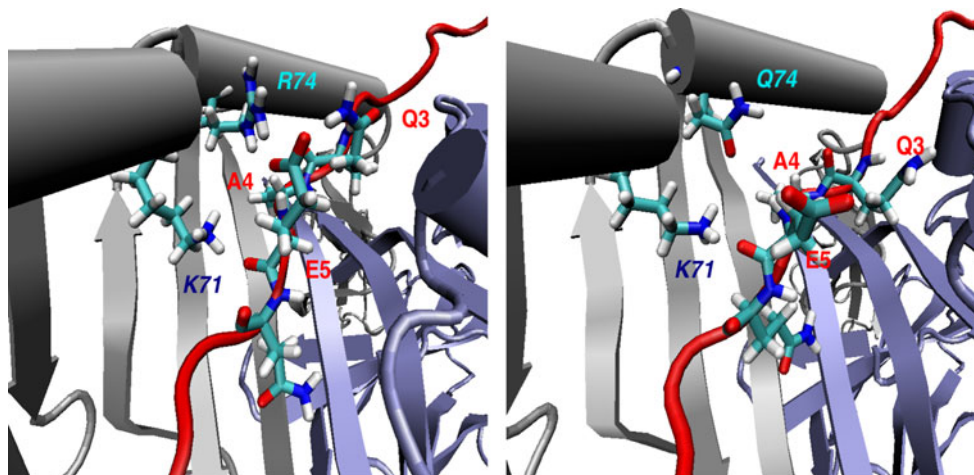


Fig. 3 Molecular illustration of the interactions between Lys- β 71 and Arg- β 74, and the Tg.726 peptide. *Left*, Arg- β 74 forms a strong salt bridge interaction with Glu-5 of the peptide and induces a strained conformation of the side chain. *Right*, R74Q β mutation weakens the interaction with Glu-5, and upon release of the strain, the side chain

turns away from Gln- β 74 (Reproduced with permission from the American Society for Biochemistry and Molecular Biology. This research was originally published in The Journal of Biological Chemistry. Jacobson et al. [21]. © The American Society for Biochemistry and Molecular Biology.)

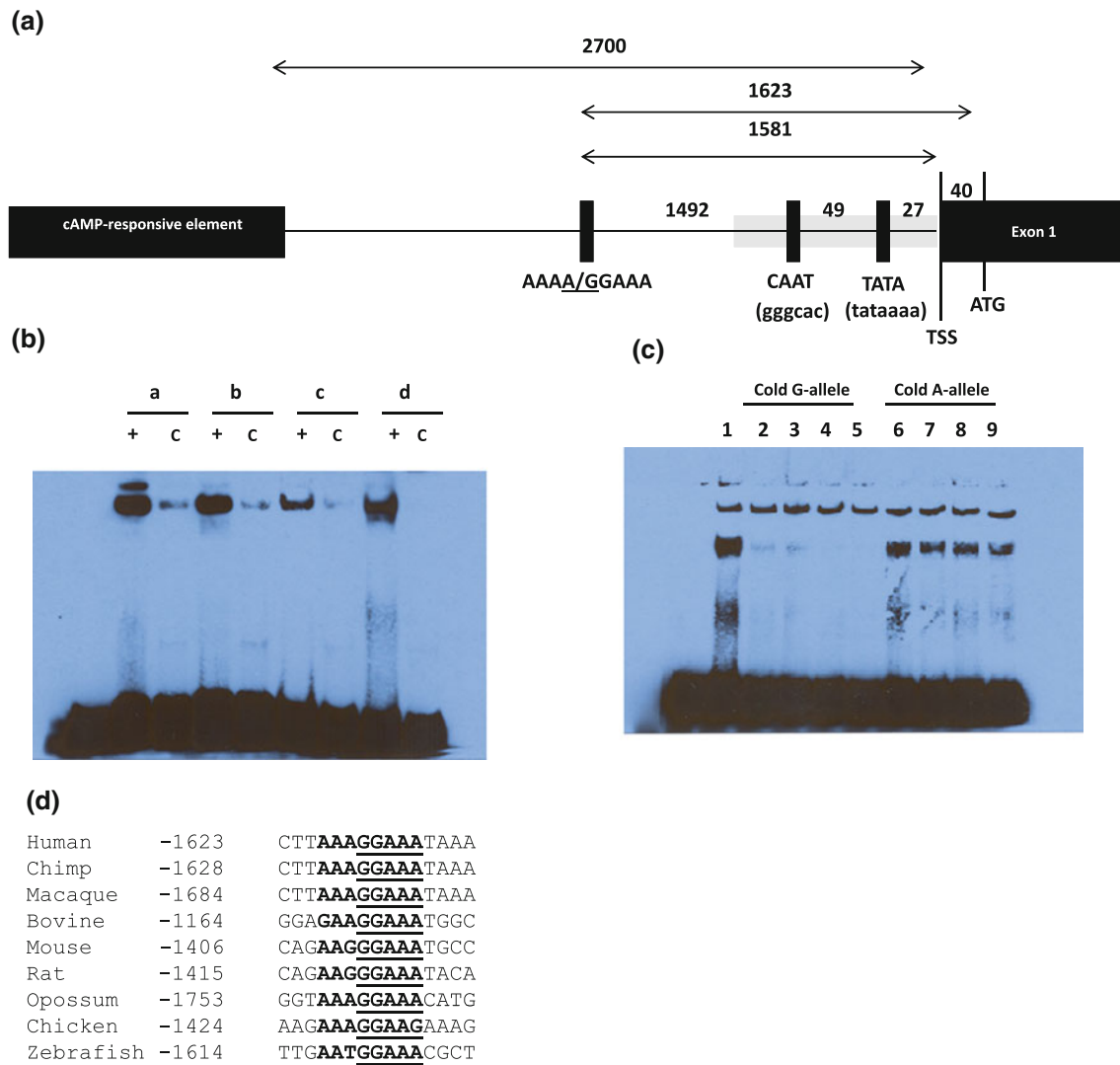


Fig. 4 rs180195 SNP is located within a transcription factor binding site. **a** Structure of the promoter and 5' upstream region of the human TG gene. **b** Electromobility shift assays (EMSA) using oligonucleotides flanking the rs180195 G variant. +, G allele with nuclear extract; C, G allele with nuclear extract and cold competitor at 200-fold excess; lane a, human placenta cell line; lane b, human brain cell line; lane c, human liver cell line; lane d, rat thyroid cell line. **c** EMSA competition experiment demonstrating specific binding of a nuclear factor to the rs180195 G allele. Nuclear extracts from human thyroid tissue were incubated with labeled dsDNA and different

concentrations of competitor. Lane 1, rs180195 G allele without competitor; lanes 2-9, rs180195 G allele with increasing concentration of G allele competitor (lanes 2-5) or A allele competitor (lanes 6-9). Only the rs180195 G allele competed off the labeled G allele. **d** Conservation of the disease-associated G polymorphism rs180195 in vertebrates (Reproduced with permission from the American Society for Biochemistry and Molecular Biology. This research was originally published in The Journal of Biological Chemistry. Stefan et al. [22]. © The American Society for Biochemistry and Molecular Biology.)

Mechanistic studies: Tg variants and the role of epigenetics

The -1623 A/G SNP at the Tg promoter (rs180195) defined a *cis*-regulatory element within the Tg promoter that was predicted to alter a transcription factor binding site. To further determine the mechanism by which this SNP contributed to disease etiology, we performed electromobility shift assays (EMSA) that demonstrated specific binding of a nuclear transcription factor to the rs180195 G

allele (associated with disease) but not to the A allele. Bioinformatic analysis predicted that either an ETS-domain protein or the interferon regulatory factor (IRF)-1 protein was the transcription factor binding to the *cis*-regulatory element containing the -1623 SNP. Chromatin immunoprecipitation (ChIP) studies using a human thyroid cell line demonstrated that the transcription factor binding to this site was IRF-1 [22].

Upon binding to the interferon (IFN) receptor, IFN α activates the transcription of IRF-1, which functions as an

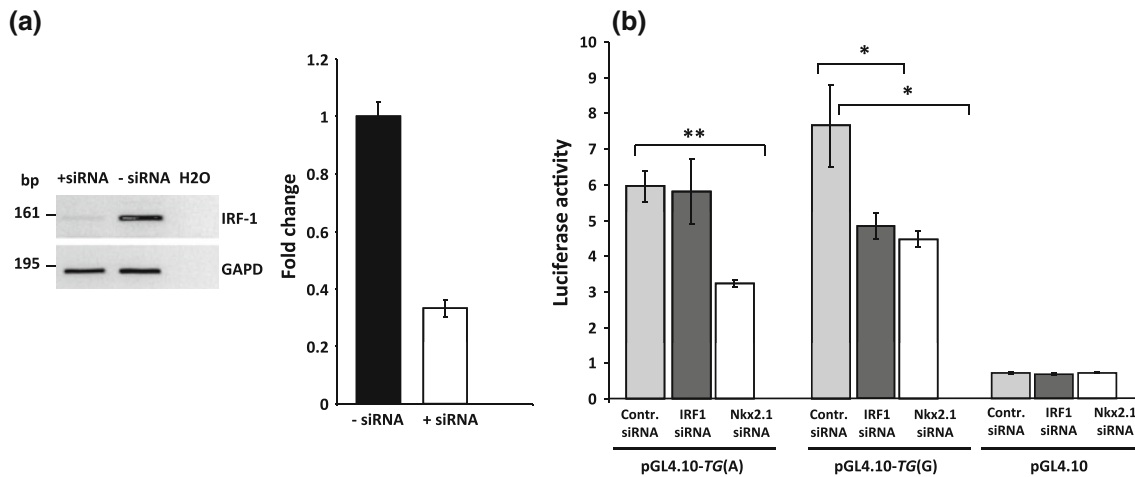


Fig. 5 **a** Reduced IRF-1 mRNA expression in ML-1 cells treated with IRF-1 siRNA. mRNA expression of IRF-1 was assessed by regular RT-PCR (*left panel*) and QRT-PCR (*right panel*). **b** Knock-down of IRF-1 by siRNA reduces the *Tg* promoter activity only in the presence of rs180195 G allele. IRF-1 siRNA decreased the relative luciferase activity by 1.5-fold ($p = 0.04$) only when the disease-associated allele (G) was present at the rs180195 site. As control, we

used siRNA to Nkx2.1 a known transcription factor driving *Tg* transcription * < 0.05 ; ** < 0.005 (Reproduced with permission from the American Society for Biochemistry and Molecular Biology. This research was originally published in *The Journal of Biological Chemistry*. Stefan et al. [22]. © The American Society for Biochemistry and Molecular Biology.)

important regulator of cellular responses to $\text{IFN}\alpha$, activating transcription of $\text{IFN}\alpha$ -inducible genes [23]. To determine whether IRF-1 indeed regulated the activity of the *Tg* promoter in an allele-dependent manner, we made constructs containing the *Tg* promoter harboring either the A or the G alleles driving the luciferase gene. When thyroid cells were transfected with these constructs, the *Tg* promoter activity was significantly higher with the G allele than with the A allele. Furthermore, when thyroid cells were co-transfected with the *Tg* promoter luciferase constructs together with short-interfering (si) RNAs targeting IRF-1, the G allele containing promoter activity was reduced to the levels of the A allele containing constructs, clearly showing that the disease-associated G allele can increase *Tg* promoter activity through binding of IRF-1 [22] (Fig. 5). Furthermore, IRF-1 binding to the rs180195 site increased *Tg* gene transcription and correlated with active chromatin structure, characterized by enrichment in histone H3 acetylation (H3Ac) and H3 monomethylation, but not trimethylation, a signature of active transcriptional enhancers [22] (Fig. 6).

These important findings support a gene–environment interaction in the development of thyroid autoimmunity, whereby conditions in which there is an increased production of $\text{IFN}\alpha$, for example viral infection, could increase expression of autoantigens such as *Tg*, and in the setting of local cytokine response, this could lead to the activation of T-cell responses to local thyroid antigens, leading to AITD in genetically susceptible individuals.

Immune-regulatory genes and AITD: CD40

Genetic studies: CD40 and the link to autoimmunity

CD40, located on chromosome 20q, is present on the surface of APCs, including B cells and plays a crucial role in B-cell differentiation and activation. Upon ligation with CD154, CD40 provides the co-stimulatory signal for B-cell proliferation, immunoglobulin class switching, antibody secretion, and the generation of memory cells (reviewed in [7]). A stimulated B cell that receives no secondary CD40 signal, for example, will not mature and will secrete only modest amounts of low-affinity antibodies, of the IgM subtype. Evidently, the CD40 molecule is crucial to both the innate and adaptive immune responses and has therefore been linked to several autoimmune disorders, including asthma and rheumatoid arthritis (reviewed in [11]). The use of antibodies against CD154, for example, was shown to successfully block the development of primary and secondary antibody responses. It also had beneficial effects on certain experimental autoimmune conditions, including experimental models of Graves' disease and myasthenia gravis (reviewed in [7]).

Through the use of linkage and association studies, we have shown that CD40 is a major susceptibility gene in GD [18, 24]. To determine the causative variant associated with GD, we performed sequencing studies and demonstrated an association of GD with a C/T polymorphism at the 5'UTR strategically located in the Kozak sequence of the CD40 gene [24, 25]. The Kozak sequence is a nucleotide

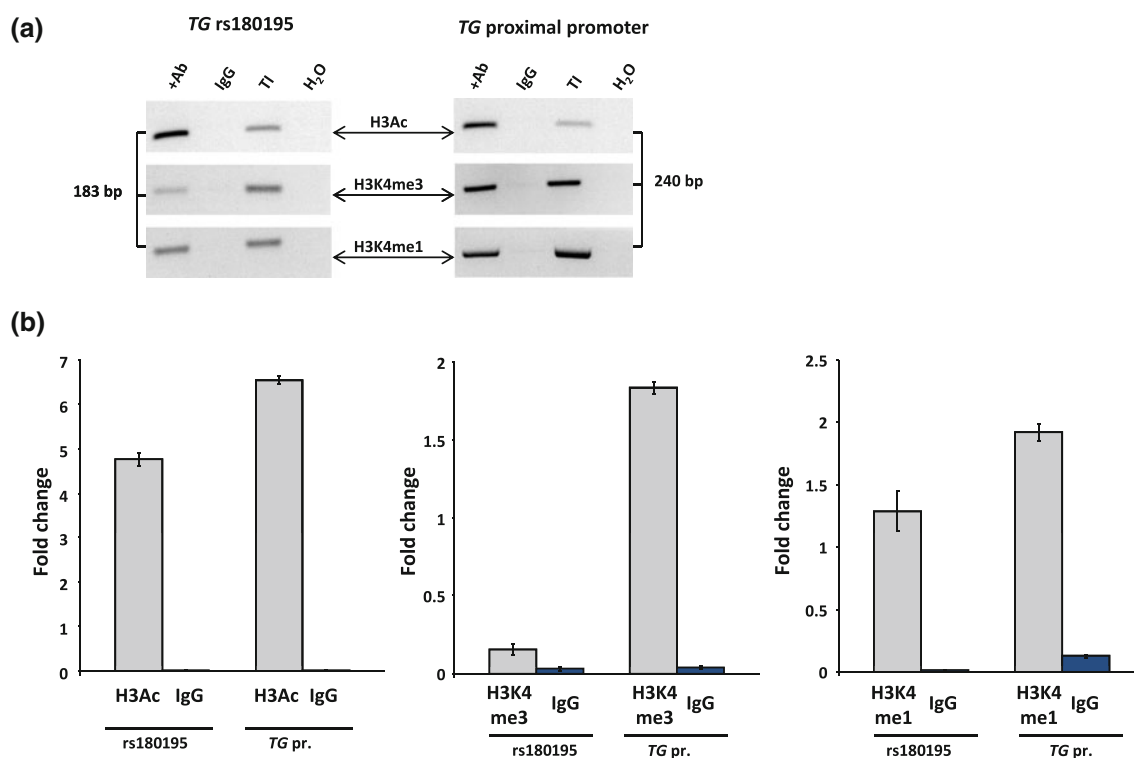


Fig. 6 Histone markers at the rs180195 site in ML-1 thyroid cells. Chromatin from ML-1 cells was immunoprecipitated with anti-acetylated histone H3 (H3Ac), anti-trimethyl-histone H3(K4) (H3K4me3), and anti-monomethyl-histone H3(K4) (H3K4me1) Abs. **a** PCR of DNA from chromatin immunoprecipitated with H3Ac, H3K4me3 and H3K4me1. PCR was done using primers for the rs180195 site and for the TG proximal promoter. **b** Quantitative PCR for the rs180195 site and for the TG proximal promoter. TG proximal promoter showed increased trimethylation and monomethylation of

histone H3 at Lys-4 residue, but the rs180195 site showed enrichment only of monomethylated H3K4, a signature of active transcriptional enhancers. Chromatin regions of both the rs180195 site and the TG proximal promoter showed elevated levels of H3Ac (Reproduced with permission from the American Society for Biochemistry and Molecular Biology. This research was originally published in *The Journal of Biological Chemistry*. Stefan et al. [22]. © The American Society for Biochemistry and Molecular Biology)

sequence essential for the initiation of translation. More specifically, the C/C genotype of this SNP was strongly associated with GD. These results were confirmed in a meta-analysis demonstrating significant association between the CC genotype and GD (reviewed in [7]). Furthermore, the data remain consistent among different ethnic groups, including Caucasians, Japanese, and Koreans (reviewed in [7]). Interestingly, we have recently shown a strong association between the CC genotype and subset of GD patients that had persistently high levels of post-treatment thyroid antibodies [26], suggesting a role for CD40 in thyroid antibody production.

Mechanistic studies: the role of CD40 Kozak sequence and its contribution to GD

The CD40 Kozak SNP has significant effects on the expression of CD40 and the subsequent risk of autoimmunity. Functionally, we have shown that the C allele increased CD40 mRNA translation by approximately 20–30 % when compared to the protective T allele [27].

Stimulated by the C allele, increased translation of CD40 can result in increased expression of CD40 on B cells and on thyroid follicular cells. This increased CD40 expression may enhance production of antibodies and activate resident T cells to trigger thyroid autoimmunity (reviewed in [7, 11]). A possible explanation is that autoreactive B cells expressing higher levels of CD40 may have a lower threshold for activation, further contributing to the onset of autoimmune disease. Studies have reported that even minor changes in the expression of B-cell surface receptors can initiate autoimmunity [28]. Likewise, we propose that even subtle changes in the levels of CD40, driven by the C allele, can lead to enhanced CD40 expression and contribute to disease etiology.

The Kozak SNP can also increase translation and expression of CD40 in target tissues, such as the thyroid itself. Indeed, CD40 has been demonstrated to be expressed and functional on thyroid cells [29]. The role of target tissue CD40 expression in the etiology of autoimmune disease, however, has not yet been fully determined. In our recent work, we examined the effects of CD40 over-expression in

the thyroid using an experimental autoimmune Graves' disease model (EAGD) (unpublished data). We showed that thyroidal CD40 over-expression in mice significantly increased levels of thyrotropin (TSH) receptor antibodies ($p = 0.026$) and thyroid hormone production (T4) ($p = 0.005$), implying that these antibodies are pathogenic in nature (unpublished results) and may ultimately augment disease severity. To further determine whether CD40 is in fact necessary for disease development, we deleted thyroidal CD40 in mice using a chimeric approach. While almost all control mice developed thyroid antibodies, the number of mice lacking thyroidal CD40 that produced antibodies was significantly lower ($p = 1.6 \times 10^{-5}$). The mice lacking thyroidal CD40 also demonstrated a decreased incidence of EAGD (unpublished data). Taken together, these data strongly suggest that thyroidal CD40 expression, in combination with other genetic and non-genetic factors, is associated with the production of pathogenic thyroid-specific antibodies [27] and can augment the severity of Graves' disease, but it is not required for disease development, as 32.5 % of mice lacking thyroidal CD40 still developed disease. To help identify potential mechanisms underlying the effects of thyroidal CD40 expression on thyroid autoimmunity, we performed transcriptome and immune-pathway analyses. Interestingly, stimulation of CD40 in human primary thyroid follicular cells activated signaling pathways for several pro-inflammatory chemokines and cytokines, most notably IL-6, which was also upregulated in the thyroids of transgenic mice with acute EAGD. In order to examine the effects of IL-6 in the setting of thyroidal CD40 over-expression, we blocked IL-6 production during induction of EAGD in mice and showed a decrease in levels of TSHR-stimulating antibodies and in frequency of disease (unpublished data). It is likely that activation of the CD40 signaling pathway in thyroid cells leads to downstream cytokine secretion (i.e., IL-6) and activation of resident T cells, ultimately promoting thyroid inflammation and autoimmunity via bystander mechanisms. In view of these findings, we concluded that target tissue expression of CD40 and its resulting cytokine response offer exciting potential therapeutic targets in Graves' disease and other organ-specific autoimmune conditions.

Epigenetics: the link between susceptibility genes and environmental triggers

While significant progress has been made in our understanding of the genetic contribution to thyroid autoimmunity, the mechanisms by which gene variants interact with environmental factors to cause autoimmunity are still unclear. Recent data suggest that epigenetic mechanisms might underlie gene–environment interactions in complex

diseases. Epigenetic effects were originally defined as heritable effects on gene expression that are not coded in the DNA sequence. However, more recently, epigenetic effects on gene expression have been broadened to include non-DNA-sequence-encoded effects on gene expression that are mitotically stable (i.e., long-lasting). Epigenetic modulation of gene expression can occur through alterations in DNA methylation, histone modification patterns (usually acetylation, de-acetylation, and methylation), and RNA interference through microRNAs [30, 31]. In several autoimmune diseases including type 1 diabetes [32], systemic lupus erythematosus [33], and rheumatoid arthritis [34], epigenetic changes have been shown to play a role in the etiology of disease. Recently, we have shown IFN α -induced alterations in thyroglobulin gene expression through epigenetic changes in histone modifications (see above). Since IFN α is secreted locally during viral infections, this could be an attractive mechanism by which infections can trigger AITD [35].

Conclusion and future directions

There have been significant advances in the mapping of AITD susceptibility genes and in the mechanisms by which they cause disease. Triggered by an intricate interaction between susceptibility genes and environmental factors, the mechanisms by which AITD develop are also slowly becoming unraveled. As demonstrated by our more recent studies, epigenetic effects offer a high potential mechanism by which disease-associated genetic variants interact with the environment to induce immune dysregulation and autoimmunity.

Acknowledgments This work was supported in part by National Institutes of Health Grants DK61659, DK067555, and DK073681. This work was also supported by a Veterans Affairs merit award (to Y.T.).

References

1. Cocks Eschler D, Hasham A, Tomer Y. Cutting edge: the etiology of autoimmune thyroid diseases. *Clin Rev Allergy Immunol.* 2011;41(2):190–7.
2. Brent GA. Environmental exposures and autoimmune thyroid disease. *Thyroid.* 2010;20(7):755–61.
3. Papanastasiou L, Vatalas IA, Koutras DA, Mastorakos G. Thyroid autoimmunity in the current iodine environment. *Thyroid.* 2007;17(8):729–39.
4. Mandac JC, Chaudhry S, Sherman KE, Tomer Y. The clinical and physiological spectrum of interferon-alpha induced thyroiditis: toward a new classification. *Hepatology.* 2006;43(4):661–72.
5. Jacobson EM, Huber A, Tomer Y. The HLA gene complex in thyroid autoimmunity: from epidemiology to etiology. *J Autoimmun.* 2008;30:58–62.
6. Tomer Y, Huber A. The etiology of autoimmune thyroid disease: a story of genes and environment. *J Autoimmun.* 2009;32:231–9.

7. Jacobson EM, Tomer Y. The CD40, CTLA4, thyroglobulin, TSH receptor, and PTPN22 gene quintet and its contribution to thyroid autoimmunity: back to the future. *J Autoimmun.* 2007;28:85–98.
8. Zamani M, Spaepen M, Bex M, Bouillon R, Cassiman JJ. Primary role of the HLA class II DRB1*0301 allele in graves disease. *Am J Med Genet.* 2000;95:432–7.
9. Golden B, Levin L, Ban Y, Concepcion E, Greenberg DA, Tomer Y. Genetic analysis of families with autoimmune diabetes and thyroiditis: evidence for common and unique genes. *J Clin Endocrinol Metab.* 2005;90(8):4904–11.
10. Menconi F, Osman R, Monti MC, Greenberg DA, Concepcion ES, Tomer Y. Shared molecular amino acid signature in the HLA-DR peptide binding pocket predisposes to both autoimmune diabetes and thyroiditis. *Proc Natl Acad Sci USA.* 2010;107(39):16899–903.
11. Tomer Y. Genetic susceptibility to autoimmune thyroid disease: past, present, and future. *Thyroid.* 2010;20(7):715–25.
12. Ban Y, Davies TF, Greenberg DA, Concepcion ES, Osman R, Oashi R, Tomer Y. Arginine at position 74 of the HLA-DR beta chain is associated with Graves' disease. *Genes Immun.* 2004;5(3):203–8.
13. Aitman TJ, Todd JA. Molecular genetics of diabetes mellitus. *Baillieres Clin Endocrinol Metab.* 1995;9:631–56.
14. Morel PA, Dorman JS, Todd JA, McDevitt HO, Trucco M. Aspartic acid at position 57 of the HLA-DQ beta-chain protects against type 1 diabetes: a family study. *Proc Natl Acad Sci USA.* 1988;85:8111–5.
15. Simmonds MJ, Howson JM, Heward JM, Cordell HJ, Foxall H, Carr Smith J, Gibson SM, Walker N, Tomer Y, Franklyn JA, Todd JA, Gough SC. Regression mapping of association between the human leukocyte antigen region and Graves disease. *Am J Hum Genet.* 2005;76(1):157–63.
16. Menconi F, Monti MC, Greenberg DA, Oashi T, Osman R, Davies TF, Ban Y, Jacobson EM, Concepcion ES, Li CW, Tomer Y. Molecular amino acid signatures in the MHC class II peptide binding pocket predispose to autoimmune thyroiditis in humans and in mice. *Proc Natl Acad Sci USA.* 2008;105(37):14034–9.
17. Hodge SE, Ban Y, Strug LJ, Greenberg DA, Davies TF, Concepcion ES, Villanueva R, Tomer Y. Possible interaction between HLA-DR β 1 and thyroglobulin variants in Graves' disease. *Thyroid.* 2006;16:351–5.
18. Tomer Y, Ban Y, Concepcion E, Barbesino G, Villanueva R, Greenberg DA, Davies TF. Common and unique susceptibility loci in Graves' and Hashimoto disease: results of whole genome screening in a data set of 102 multiplex families. *Am J Hum Genet.* 2003;73(4):736–47.
19. Sakai K, Shirasawa S, Ishikawa N, Ito K, Tamai H, Kuma K, Akamizu T, Tanimura M, Furugaki K, Yamamoto K, Sasazuki T. Identification of susceptibility loci for autoimmune thyroid disease to 5q31–q33 and Hashimoto's thyroiditis to 8q23–q24 by multipoint affected sib-pair linkage analysis in Japanese. *Hum Mol Genet.* 2001;10(13):1379–86.
20. Ban Y, Greenberg DA, Concepcion ES, Skrabanek L, Villanueva R, Tomer Y. Amino acid substitutions in the thyroglobulin gene are associated with susceptibility to human and murine autoimmune thyroid disease. *Proc Natl Acad Sci USA.* 2003;100:15119–24.
21. Jacobson EM, Yang H, Menconi F, Wang R, Osman R, Skrabanek L, Li CW, Fadlalla M, Gandhi A, Chaturvedi V, Smith EP, Schwemberger S, Osterburg A, Babcock GF, Tomer Y. Employing a recombinant HLA-DR3 expression system to dissect major histocompatibility complex II-thyroglobulin peptide dynamism: a genetic, biochemical, and reverse immunological perspective. *J Biol Chem.* 2009;284(49):34231–43.
22. Stefan M, Jacobson EM, Huber AK, Greenberg DA, Li CW, Skrabanek L, Concepcion E, Fadlalla M, Ho K, Tomer Y. Novel variant of thyroglobulin promoter triggers thyroid autoimmunity through an epigenetic interferon α -modulated mechanism. *J Biol Chem.* 2011;286(36):31168–79.
23. Taniguchi T, Ogasawara K, Takaoka A, Tanaka N. IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol.* 2001;19:623–55.
24. Tomer Y, Concepcion E, Greenberg DA. A C/T single nucleotide polymorphism in the region of the CD40 gene is associated with Graves' disease. *Thyroid.* 2002;12(12):1129–35.
25. Ban Y, Tozaki T, Taniyama M, Tomita M, Ban Y. Association of a C/T single nucleotide polymorphism in the 5' untranslated region of the CD40 gene with Graves' disease in Japanese. *Thyroid.* 2006;16(5):443–6.
26. Jacobson EM, Huber AK, Akeno N, Sivak M, Li CW, Concepcion E, Ho K, Tomer Y. A CD40 Kozak sequence polymorphism and susceptibility to antibody-mediated autoimmune conditions: the role of CD40 tissue-specific expression. *Genes Immun.* 2007;8(3):205–14.
27. Jacobson EM, Concepcion E, Oashi T, Tomer Y. A Graves' disease-associated kozak sequence single-nucleotide polymorphism enhances the efficiency of CD40 gene translation: a case for translational pathophysiology. *Endocrinology.* 2005;146(6):2684–91.
28. Sato S, Hasegawa M, Fujimoto M, Tedder TF, Takehara K. Quantitative genetic variation in CD19 expression correlates with autoimmunity. *J Immunol.* 2000;165:6635–43.
29. Metcalfe RA, McIntosh RS, Marelli-Berg F, Lombardi G, Lechler R, Weetman AP. Detection of CD40 on human thyroid follicular cells: analysis of expression and function. *J Clin Endocrinol Metab.* 1998;83(4):1268–74.
30. Jungel A, Ospelt C, Gay S. What can we learn from epigenetics in the year 2009? *Curr Opin Rheumatol.* 2010;22(3):284–92.
31. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. High-resolution profiling of histone methylations in the human genome. *Cell.* 2007;129(4):823–37.
32. Bell CG, Teschendorff AE, Rakyan VK, Maxwell AP, Beck S, Savage DA. Genome-wide DNA methylation analysis for diabetic nephropathy in type 1 diabetes mellitus. *BMC Med Genomics.* 2010;3:33.
33. Heerwagen MJ, Miller MR, Barbour LA, Friedman JE. Maternal obesity and fetal metabolic programming: a fertile epigenetic soil. *Am J Physiol Regul Integr Comp Physiol.* 2010;299(3):R711–22.
34. Karouzakis E, Gay RE, Gay S, Neidhart M. Epigenetic control in rheumatoid arthritis synovial fibroblasts. *Nat Rev Rheumatol.* 2009;5(5):266–72.
35. Youngblood B, Reich NO. The early expressed HIV-1 genes regulate DNMT1 expression. *Epigenetics.* 2008;3(3):149–56.