

Joint Genetic Susceptibility to Type 1 Diabetes and Autoimmune Thyroiditis: from Epidemiology to Mechanisms

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Type 1 diabetes (T1D) and autoimmune thyroid diseases (AITD) frequently occur together within families and in the same individual. The co-occurrence of T1D and AITD in the same patient is one of the variants of the autoimmune polyglandular syndrome type 3 [APS3 variant (APS3v)]. Epidemiological data point to a strong genetic influence on the shared susceptibility to T1D and AITD. Recently, significant progress has been made in our understanding of the genetic association between T1D and AITD. At least three genes have been confirmed as major joint susceptibility genes for T1D and AITD: human leukocyte antigen class II, cytotoxic T-lymphocyte antigen 4 (CTLA-4), and protein tyrosine phosphatase non-receptor type 22. Moreover, the first whole genome linkage study has been recently completed, and additional genes will soon be identified. Not unexpectedly, all the joint genes

for T1D and AITD identified so far are involved in immune regulation, specifically in the presentation of antigenic peptides to T cells. One of the lessons learned from the analysis of the joint susceptibility genes for T1D and AITD is that subset analysis is a key to dissecting the etiology of complex diseases. One of the best demonstrations of the power of subset analysis is the CTLA-4 gene in T1D. Although CTLA-4 showed very weak association with T1D, when analyzed in the subset of patients with both T1D and AITD, the genetic effect of CTLA-4 was significantly stronger. Gene-gene and genetic-epigenetic interactions most likely play a role in the shared genetic susceptibility to T1D and AITD. Dissecting these mechanisms will lead to a better understanding of the etiology of T1D and AITD, as well as autoimmunity in general. (*Endocrine Reviews* 29: 697–725, 2008)

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I. Introduction

THE AUTOIMMUNE ENDOCRINE diseases are organ specific autoimmune diseases in which the target organs are endocrine glands such as the thyroid, adrenal, islet cells, and ovaries. The most common autoimmune endocrine disorders are type 1 diabetes (T1D) and the autoimmune thyroid diseases (AITD). T1D and AITD are both characterized by T cell infiltration and production of autoantibodies directed at the target organs (pancreatic islets and the thyroid, respectively), resulting in their dysfunction or destruction. Intriguingly, autoimmune endocrine disorders fre-

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Abbreviations: AITD, autoimmune thyroid disease; APC, antigen-presenting cell; APS, autoimmune polyglandular syndrome; APS3v, APS3 variant; CTLA-4, cytotoxic T-lymphocyte antigen 4; DZ, dizygotic; FOXP3, forkhead box P3; GD, Graves' disease; HLA, human leukocyte antigen; HT, Hashimoto's thyroiditis; ICA, islet cell antibodies; INS, insulin gene; LD, linkage disequilibrium; LOD, logarithm of odds; LYP, lymphoid tyrosine phosphatase; MHC, major histocompatibility complex; MICA, MHC class I chain related gene; MLS, maximum LOD score; MZ, monozygotic; PTPN22, protein tyrosine phosphatase non-receptor type 22; RR, relative risk; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism; TAb, thyroid antibodies; T1D, type 1 diabetes; TDT, transmission disequilibrium test; Tg, thyroglobulin; TPO, thyroid peroxidase; TSHR, TSH receptor; UTR, untranslated region; VNTR, variable number of tandem repeats.

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quently occur together in the same individual, and this association is classified as an autoimmune polyglandular syndrome (APS) (1). Of all the associations between autoimmune endocrine disorders, by far the most common is between T1D and AITD (2) (please note that in this review the abbreviation AITD includes GD, and HT). Up to 20% of patients with T1D have thyroid antibodies (TAb), with 50% of these progressing to clinical AITD (3). Conversely, 2.3% of children with AITD have islet cell antibodies (ICA) compared with 0% of controls (4). Although the exact mechanisms of this association are still evolving, there is growing evidence that genetic factors play a major role. In this review, we will discuss the genetics of T1D and AITD, focusing on the joint genetic factors contributing to the genetic association of these two disorders.

II. Evidence for a Shared Genetic Susceptibility to T1D and AITD

A. Evidence for genetic susceptibility to T1D

1. *Population data.* Epidemiological surveys have been conducted analyzing the prevalence and incidence rates of T1D worldwide. Large differences in the incidence of T1D have been reported in different ethnic and geographic groups around the globe. In a large study from the DIAMOND project group, the trends in age- and sex-specific incidence of T1D between 1990 and 1999 were examined in 57 countries worldwide (5–9). This survey showed a wide variation in the incidence of T1D across populations, ranging from 0.1 per 100,000/year in China and Venezuela to 40.9 per 100,000/year in Finland (10, 11).

These significant differences in the prevalence of T1D across many different populations around the globe may be due to environmental factors to which these populations are exposed (*e.g.*, diet, infections) and/or genetic factors because there are ethnic/genetic differences between the populations studied. Most likely, both environment and genetics contribute to this worldwide variation in T1D frequency. Supporting a genetic effect are studies showing variation in T1D prevalence in different ethnic groups living in the same geographic region. For example, there is a significant difference in the incidence of T1D among African-Americans and Caucasians living in the United States, with prevalence among Caucasians being much higher than in African-Americans (12). Similarly, there is a wide variation in the prevalence of T1D among Arabs and Jews living in Israel, with Jews having a higher prevalence of the disease (13, 14). Such variations among different ethnic groups living in the same region support a genetic influence on the prevalence of the disease. On the other hand, other studies have shown differences in T1D frequency between ethnically homogenous populations living in different geographic regions, demonstrating that environmental factors contribute to these geographic differences as well. For example, surveys in Nordic countries have shown a two to four times higher frequency of T1D in Finland, Sweden, and Norway, compared with Estonia and Iceland (7, 8, 15, 16).

2. *Family studies.* Familial clustering of a disease suggests genetic influences on the etiology. Indeed, familial aggrega-

tion of T1D has been reported in many studies (17–21). In Caucasians, T1D clusters in families, shown by the observation that there is a higher lifetime risk in siblings of T1D probands (6%) compared with the frequency in the general population (0.4%) (22, 23). Interestingly, even in countries with low incidence of T1D, familial clustering of the disease has been observed. In a nationwide study of multiplex families from Japan, a country with low incidence of T1D, it was found that the frequency of T1D among siblings of diabetic probands was 1.3–3.8% compared with a very low frequency in the general population of 0.014% (21). This sibling frequency of T1D in Japanese families is slightly lower but similar to that seen in Caucasians (6%) (19, 20), suggesting that even in low incident countries, such as Japan, there is significant familial clustering of T1D. This supports a strong genetic influence on the development of T1D. Interestingly, the risk of T1D in first- and second-degree relatives declined in a pattern suggesting a multiplicative effect of more than one gene (24).

3. *Sibling risk ratio (λ_s).* Familial clustering of a disease can be due to nongenetic factors, such as the shared environmental exposures (*e.g.*, infections, diet). Therefore, several methods have been developed to determine whether familial clustering of a disease is the result of genetic susceptibility or nongenetic factors. One method is to calculate the sibling risk ratio (λ_s), which expresses the increased risk of developing the disease in an individual who has a sibling with the disease compared with the risk in the general population and is a quantitative measure of the genetic contribution to the disease (25). A λ_s greater than 5 usually indicates a significant genetic contribution to the pathogenesis of a disease (26). In Caucasians, the risk to a sibling of a T1D patient of developing disease is 6% compared a 0.4% risk of T1D in the general population. This results in a λ_s of 6/0.4 or 15, supporting a strong genetic component in the etiology of the disease (27). Intriguingly, in the Japanese population, the λ_s for T1D ranges from 93 to 271 using the percentages from the above mentioned family studies (1.3–3.8%/0.014%) (21). Thus, it is possible that in countries with low frequency of T1D the genetic component in the etiology of the disease is even stronger, but the gene frequency in the population is low.

4. *Twin studies.* Twin studies are based on comparison of the concordance (simultaneous occurrence) of a given disease among monozygotic (MZ; *i.e.*, identical) twins with the concordance among dizygotic (DZ; *i.e.*, fraternal) twins. MZ twins have identical genetic makeup, whereas DZ twins share an average of 50% of their genes. Therefore, if the concordance is higher in the MZ twins compared with the DZ twins, it suggests that the disease has an inherited component. Several twin studies have shown a higher concordance rate of T1D in MZ twins when compared with DZ twins. In MZ twins, the rate of T1D ranges from 13–67.7% with an average of 46.2% (Table 1), compared with a range of 0–12.4% in DZ twins (average of 8.4%, Table 1) (28–36) (also reviewed in Ref. 21). In addition, the concordance rate for positive T1D autoantibodies (cytoplasmic islet cell, insulin, glutamic acid decarboxylase 65, and ICA512) was higher in

TABLE 1. Selected twin studies in T1D

First author (Ref.)	Monozygotic twins			Dizygotic twins		
	Affected(n)	Total(n)	%	Affected(n)	Total(n)	%
Gottlieb (28)	9	30	30	2	70	2.9
Harvald (29)	38	83	45.8	22	158	13.9
Tattersall (34)	65	96	67.7	N/A	N/A	N/A
Barnett (35)	80	147	54.4	N/A	N/A	N/A
Matsuda (30)	9	19	47.3	1	13	7.6
Leslie (31)	113	211	53.5	0	21	0
Kumar (32)	38	132	28.9	13	105	12.4
Kaprio (33)	3	23	13	2	81	2.4
Redondo (37)	12	53	22.3	0	30	0
Total	367	794	46.2	40	478	8.4

N/A, Not available.

MZ twins (34, 25, 37, and 14%, respectively) compared with DZ twins (10, 7, 13, and 4%, respectively) (37). This, too, suggests an important genetic component in the etiology of islet cell autoimmunity.

B. Evidence for genetic susceptibility to AITD

1. Population data. Epidemiological surveys have shown relatively similar prevalence and incidence rates of Graves' disease (GD) in Caucasian populations of different iodine-sufficient geographic origins (38–44). Similar prevalence and incidence trends across geographic regions were also observed for Hashimoto's thyroiditis (HT) (38, 39, 45–47). The comparable prevalence and incidence of GD and HT (*i.e.*, AITD) in geographically different populations suggests a significant genetic effect on the development of the disease because these populations are exposed to different environmental factors. Longitudinal surveys also suggest a strong genetic component in the etiology of AITD. A longitudinal study from the Mayo clinic (1935–1967) showed no significant change in the incidence of GD over the 33 yr of the study (48). The stable incidence of GD with time points to strong genetic effects because the genetic makeup of a population does not change over several decades, but environmental factors usually do. The Mayo Clinic observations were supported by a more recent study from Sweden (49). However, the Swedish study found an increased incidence of GD in a subset of the population, demonstrating that environmental effects also play a role in the etiology of GD. In the Mayo survey (1935–1967) there was a significant increase in the incidence of HT over the 33 yr of the survey (48). This could reflect a stronger environmental influence on the development of HT or a change in the diagnostic criteria over time (50).

2. Family studies. The familial occurrence of AITD has been reported by investigators for many years (51–53). There are many studies showing a high frequency of thyroid abnormalities in relatives of patients with GD (54–57), most commonly the presence of thyroid autoantibodies, which were reported in up to 50% of the siblings of patients with GD (55, 58, 59). A recent survey by our group revealed that 36% of GD patients with ophthalmopathy had a family history of AITD, whereas 32% had a first-degree relative with AITD (60).

3. Sibling risk ratio (λ s). As mentioned above, the λ s is a useful quantitative measure of the heritability of a disease, with a λ s greater than 5 usually indicating a genetic influence on the etiology of the disease (25, 26). We have calculated the λ s in AITD in a cohort of 155 AITD patients. The λ s was 16.9 for AITD, 11.6 for GD, and 28.0 for HT, indicating a strong genetic influence on the development of AITD.

4. Twin studies. Several groups have performed twin studies in AITD. All of these studies have shown a significantly higher concordance of AITD in MZ twins when compared with DZ twins. For GD, the concordance rates were 35% in MZ twins and 3% in DZ twins (61–63). For HT, the concordance rates were 55 and 0% for MZ and DZ twins, respectively (64); for TAb (64, 65), MZ twins had 80% concordance, and DZ twins had 40% concordance (64). Thus, the twin data support a substantial inherited susceptibility to AITD.

C. Shared genetic susceptibility to T1D and AITD

1. Population data. Several studies across different populations have shown, using serology for thyroid and islet cell antibodies, that there is a frequent co-occurrence of T1D and AITD within the same individuals. In most of these studies, researchers analyzed the occurrence of two thyroid-specific antibodies [anti-thyroid peroxidase (anti-TPO) and anti-thyroglobulin (anti-Tg)] in T1D patients as an indicator of thyroid autoimmunity. [Please note that in this review the abbreviation TAb refers to the presence of anti-TPO antibodies, anti-Tg antibodies, or both; it does not include TSH receptor antibodies.] The frequency of TAb in T1D patients varied among studies from 8% to as high as 44% (66–72). However, even the lowest frequency reported is still significantly higher than the prevalence of TAb in age-matched controls (67). One study examined the reverse phenomenon, namely the frequency of ICA among AITD patients. This study showed that 2.3% of AITD children had ICA, compared with 0% of control children (4). In the same study, 30% of T1D children had TAb compared with 4.3% of controls (4). The increased frequency of TAb in children with T1D has been consistent across different populations. In Germany and Austria, 10 to 21.6% of T1D patients tested positive for one or both TAb, compared with 0 to 3.7% of the general population (3, 73, 74). Interestingly, in one study (74), a follow-up on 16 patients with T1D showed that in an average of 3.5 yr after first detection of TAb, eight (50%) patients had devel-

oped thyroid disorders, and in another study (3) 16% of T1D patients having thyroid autoimmunity, as determined by elevated TAB levels, had elevated TSH levels indicating clinical AITD. Similarly, a study done in northern Europe on 105 individuals showed that 16.2% of T1D patients were TAB positive (75). In addition, a study by Burek *et al.* (12) examined the frequency of AITD in African-American compared with Caucasian children with T1D in the United States. They showed that AITD was more prevalent in Caucasian children with T1D than in African-Americans; however, the prevalence of TAB in both Caucasians (50%) and African-Americans (16%) was higher than in the general population (12). Finally, in Brazil, a study done in 383 T1D patients showed that 64 (16.7%) tested positive for TAB, with positive subjects being predominantly females (76). In addition to gender, age seems to play an important role in the onset of AITD in T1D patients. In a study by Holl *et al.* (73), 495 T1D patients were screened for TAB at multiple time points. The screening demonstrated that the prevalence of TAB in T1D patients increased dramatically with age, from 3.7% (at ages < 5 yr) to 25.3% (at ages 15–20) (73).

In summary, the increased prevalence of TAB among children with T1D is a consistent phenomenon across geographically and ethnically distinct populations, albeit the frequency of positive TAB in T1D patients varies in different populations. The frequency of TAB in T1D increases with age and is more common in females than in males. Taken together, these epidemiological observations support a strong genetic association between T1D and AITD. Indeed, the association between T1D and AITD is considered one of the variants of the APS type 3 (77, 78), and we shall refer to it in this review as APS3 variant (APS3v).

2. Family studies. The strongest epidemiological support for the existence of a shared genetic susceptibility to T1D and AITD comes from family studies. In one study from Germany, 11.6% of the first-degree relatives of T1D patients had ICA, and 7.8% had either anti-TPO or anti-Tg antibodies, compared with a prevalence of 4.0% ICA and 3.2% TAB in healthy controls (79). Similarly, a group from Greece reported that of 429 healthy first-degree relatives of T1D patients, 91 (21.2%) tested positive for TAB whereas 36 (8.39%) tested positive for ICA antibodies (80). Similar findings were also reported in non-Caucasians. Burek *et al.* (12) compared the frequency of TAB in siblings of T1D patients in Caucasian and African-American families. In both Caucasian and African-American families, the frequency of TAB was significantly increased in siblings of T1D patients (48 and 35%, respectively) indicating that this association is not unique to Caucasian T1D families (12). In another study from Colombia, examining the familial aggregation of autoimmune diseases, there was a significant increase in the prevalence of ICA and TAB in first-degree relatives of T1D patients (81). The prevalence rates of ICA and TAB in first-degree relatives of T1D patients were 2.6 and 4.8%, respectively, compared with 0 and 1.7%, respectively, in controls (81). Moreover, in the same study it was noted that autoimmune hypothyroidism (*i.e.*, HT) was the most common thyroid disease among first-degree relatives of the T1D patients (81).

One of the largest family studies looking at the aggregation

of T1D and AITD was the Familial Autoimmune Diabetes Study (82, 83). This family study looked at prevalence rates of AITD in children with T1D and their first-degree relatives (siblings and parents). HT was commonly found in both the probands and their relatives, especially among women. Among female diabetic probands, HT was diagnosed in 54–75% of cases depending on age, and among female relatives the frequency of HT was 22–44%. Moreover, diabetic probands with HT were significantly more likely to have a family history of thyroid disease (82). Recently, another large family study from the United Kingdom also demonstrated a strong familial aggregation of T1D and AITD (84). Taken together, these data imply that the genetic association between T1D and AITD is even stronger in familial T1D. Therefore, some investigators have suggested screening for AITD in all first-degree relatives of newly diagnosed T1D patients, in addition to screening the patients themselves (79).

III. Tools for Identifying Complex Disease Genes

Based on the abundant epidemiological evidence for a strong genetic effect on the development of complex diseases such as T1D and AITD, significant efforts have been made in the last 15 yr to identify susceptibility genes for complex diseases. Although many analytical methods have been used, the two basic strategies used for mapping complex disease genes are based on linkage and association studies. Both of these methods can be applied to candidate genes or to the entire human genome. Indeed, significant progress has been made in identification of complex disease genes using these tools. Below we summarize the current tools for mapping complex disease genes.

A. Linkage and association

1. Linkage. Genetic linkage techniques are powerful tools for analyzing complex disease-related genes because they detect genes that have a major influence on the development of a disease (85). However, linkage studies are less sensitive than association studies because they do not detect genes with smaller effects (85). Because less influential genes are also important, linkage and association studies are complementary and should both be used for identifying complex disease genes. The principle of linkage analysis is based on the fact that if two genes or markers are close together on a chromosome, they will cosegregate because the likelihood that a recombination will occur between them during meiosis is low. Therefore, if a tested marker is close to a disease susceptibility gene, its alleles will cosegregate with the disease in families. The logarithm of odds (LOD) score is the measure of the likelihood of linkage between a disease and a genetic marker (86). The LOD score is the base-10 LOD ratio in favor of linkage. The classical linkage tests are model based (parametric), *i.e.*, different modes of inheritance and penetrance have to be tested when calculating the likelihood of linkage. The parametric tests are the most powerful statistical tests for linkage (87, 88), and they can be used to test for heterogeneity within a dataset; heterogeneity exists in a dataset when more than one gene causes the same disease phenotype (89). In complex diseases, the mode of inheritance is often unknown,

and therefore, model-independent methods (nonparametric) have also been widely used (25). One such method is sib-pair analysis (25). In this method, siblings that are both affected by the disease being studied are tested for sharing of alleles at a marker locus. By random chance alone, the sibs would be expected to share one allele about 50% of the time and two alleles 25% of the time. If affected sib-pairs share a significantly higher than expected proportion of alleles at the marker locus, this suggests that the region containing the marker locus also contains the disease gene. The observed to expected allele sharing can then be converted to a LOD score equivalent.

In simple Mendelian disorders, a maximum LOD score (MLS) greater than 3 [*i.e.*, odds ratio greater than 1000] is considered strong evidence for linkage (86). However, the inheritance of complex diseases (*e.g.*, AITD) does not follow a simple Mendelian pattern. These diseases are likely to be caused by several genes with reduced penetrance (*i.e.*, not all the individuals inheriting the gene will develop the disease), and genetic heterogeneity also plays a role. This results in non-Mendelian transmission of the disease in pedigrees and makes mapping the susceptibility genes for complex diseases difficult. Therefore, the cutoff LOD score in complex diseases is lower than in Mendelian disorders (90). According to well-accepted guidelines, in complex diseases a LOD score greater than 1.9 is suggestive of linkage and a LOD score greater than 3.3 indicates significant linkage in studies using the parametric approach. For nonparametric sib-pair studies, the cutoff LOD scores are higher (90). Linkage is confirmed if evidence for linkage is replicated in two or more separate datasets (90). Conversely, a LOD score lower than -2.0 has been used to exclude linkage. The main advantage of linkage analysis is that it enables mapping major genes; however, minor genes and genes with modifying effects may not always be identified by linkage analysis.

2. Phenotype definitions and genetic heterogeneity. Phenotype definitions are important in genetic studies because different phenotypes are likely to be caused by different genes, and analyzing them together would make identification of these genes more difficult. For example, the phenotype of T1D may have subsets based on age of onset of disease, levels, and epitope specificity of anti-ICA, or the presence of other autoimmune diseases such as AITD. It is possible that each of these subsets is influenced by a different set of susceptibility genes, and therefore analyzing them separately is crucial. For example, we (91) and others (92, 93) have recently shown that only the subset of T1D patients that also have concurrent AITD is influenced by the cytotoxic T lymphocyte antigen 4 (CTLA-4) gene (see Section V.B).

Even when subsets of patients with uniform phenotypes are analyzed, genetic heterogeneity can still exist. Genetic heterogeneity exists when different genotypes give rise to indistinguishable phenotypes (94). If heterogeneity exists in a dataset of families/patients, the dataset may include only a subset of families/patients that are linked/associated with a tested marker. Linkage analyses have the advantage that they can be used to test for heterogeneity in a dataset when subsetting by phenotype isn't obvious. This is done using the Admixture Test, which calculates the likelihood that a por-

portion of the families in a dataset are linked to the marker (89, 95, 96). The obtained LOD score is designated heterogeneity LOD score (97). Indeed, most of the loci we found to be linked with AITD showed significant genetic heterogeneity (98).

3. Association. Linkage studies are excellent for screening the whole genome for major genes/loci. However, they have limited resolution (~ 2 – 3 million base pairs) because as the linkage interval is narrowed all markers in the region will be linked (85). Association studies are more sensitive than linkage studies and may detect minor susceptibility genes contributing less than 5% of the total genetic contribution to a disease (99).

Association analyses are performed by comparing the frequency of the allele studied (*e.g.*, HLA-DR3) in unrelated patients and in unrelated, ethnically matched controls. If the allele tested is associated with the disease, it will appear significantly more frequently in patients than in controls. The probability of having the disease in an individual positive for the allele compared with an individual negative for the allele is estimated by the odds ratio (100). There are at least two possible explanations for the existence of an association between an allele and a disease: 1) the associated allele itself is the genetic variant causing an increased risk for the disease; and 2) the associated allele itself is not causing the disease but rather a gene in linkage disequilibrium (LD) with it (101). LD exists when chromosomes with the mutant allele at the disease locus carry certain marker alleles more often than expected (see Fig. 1).

4. Family-based association studies. The population-based association method may produce spurious associations if the patients and controls are not accurately matched ("population stratification") (102). Therefore, additional association tests have been developed that are family based and use an internal control group from within each family, thus avoiding the necessity to match patients and controls altogether. The most widely used family-based association test is the transmission disequilibrium test (TDT) (102–104). The TDT is based on comparison of parental marker alleles that are transmitted and those that are not transmitted to affected children. To perform the TDT, one needs to assemble a dataset of families each consisting of at least the two parents and one affected child. Assuming two heterozygous parents for a certain tested marker, the four parental alleles in each family are categorized into two groups: those transmitted to a child with the disease (T alleles), and those not transmitted to an affected child (N alleles). The same allele may belong to the T group or the N group in different families. The frequency of the T alleles *vs.* the N alleles is then compared by a χ^2 test. An association between a certain allele and the disease exists when there is an excess occurrence of this allele in the T group compared with the N group. Although TDT analysis has the advantage that population stratification is not an issue, its main limitation is the necessity to enroll a large number of families (to ensure enough power for the analysis), which often is not feasible.

B. Markers used in linkage and association studies

1. *Microsatellites.* Microsatellites are regions in the genome that are composed of short sequence repeats, most commonly two-base CA repeats (105). Microsatellite loci are highly polymorphic (*i.e.*, have many alleles) because the number of repeats in each individual is variable. Moreover, they are uniformly distributed throughout the genome at distances of less than 1 million base pairs (105). The main advantage of microsatellites is that, in contrast to single nucleotide polymorphisms (SNPs), which are biallelic, they have several alleles. This makes them highly informative, especially in linkage studies. Therefore, microsatellites serve as excellent markers in whole genome linkage studies (see below).

2. *SNPs.* SNPs are single base pair positions in genomic DNA at which different sequence alternatives (alleles) exist in normal individuals. Although four alleles are theoretically possible (A, C, T, G), in humans most SNPs are diallelic (reviewed in Ref. 106). SNPs are very abundant, and their frequency is about one SNP per 1000 bp or less (107). Because SNPs are less informative than microsatellites (SNPs have only two alleles and microsatellites usually have more than five alleles), more SNPs are needed than microsatellites to screen a locus or the entire human genome. However, because SNPs are much more abundant and closely spaced than microsatellites, they are ideal for fine mapping genes in linked regions using association studies. The importance of SNPs stems from the fact that many have the potential to change gene function by altering amino acid sequences (nonsynonymous SNPs) and regulatory regions [*e.g.*, promoter, 5' untranslated region (UTR), 3'UTR]. Moreover, recently it has been shown that even intronic and intergenic SNPs can affect gene expression and function (108). Thus, if a SNP allele inside or near a gene is found to be significantly associated with a disease, it may be the actual causative allele, increasing susceptibility to the disease (109).

C. Candidate gene analysis

Candidate genes are genes of known sequence and location that, by virtue of their physiological functions, may be involved in disease pathogenesis. For example, mutations in the glucokinase genes were found to be the cause of maturity onset diabetes of the young (MODY) 2 (110). This gene was tested because its function in the glucose-sensing mechanism made it a candidate gene for MODY 2 (110). Because both T1D and AITD are organ-specific autoimmune diseases in which different target organs are involved, it is likely that shared susceptibility genes for T1D and AITD will be immune regulatory genes and not target organ genes. Indeed, so far all the joint susceptibility genes for T1D and AITD identified are involved in immune regulation (see Section V) (91, 111).

D. Genome-wide screens

A more powerful approach is to screen the whole human genome for linkage or association with a disease without any assumptions on disease pathogenesis (*i.e.*, a reverse-genetic

approach) (112, 113). Whole genome screening is performed by testing a panel of markers that span the entire human genome for linkage/association with a disease in a given dataset.

1. *Whole genome linkage studies in families.* Here a panel of markers, spanning the entire human genome at distances of approximately 10 cM (about 10 Mb), are tested for linkage with a disease in a dataset of families. If one or more of the markers shows evidence for linkage with the disease according to the guidelines of Lander and Kruglyak (90), these regions may harbor susceptibility genes for the disease studied. These linked regions can then be fine-mapped, and the genes identified. Whole genome linkage studies have been successful in identifying complex disease genes such as the NOD2 gene in Crohn's disease (114) and the Tg gene in AITD (115). However, because only major susceptibility genes can be identified by linkage and less influential genes may not be detected, whole genome association studies were needed.

2. *Genome-wide association studies.* Until recently, genome-wide association studies were not feasible because reliable markers spanning the entire human genome at short intervals were not available. Genome-wide association studies became a reality with the publication of the HapMap of the human genome. The HapMap project genotyped about 1 million SNPs spanning the entire human genome, at average intervals of 5 Kb, in four populations—Africans, Caucasians, Chinese, and Japanese—and tested them for haplotypes and LD. SNP haplotypes are specific combinations of alleles of SNPs that are located on the same chromosome. LD is the nonrandom association of alleles (Fig. 1). If one looks at two SNPs, A and B, each having two alleles, *i.e.*, A1 and A2, and B1 and B2, respectively, there are four possible combinations of alleles (or haplotypes) at these two loci, A1B1, A1B2, A2B1, and A2B2. The expected frequency of each of these possible haplotypes is calculated by multiplying the frequencies of the two alleles in the population. If a certain haplotype (*e.g.*, A1B1) appears significantly more frequently than expected, then the two alleles are in LD (Fig. 1).

Several statistical tests (*e.g.*, the D' and r^2 tests) can analyze marker genotypes in a dataset for LD. The findings of the HapMap project were remarkable. The HapMap project has demonstrated that approximately 80% of the human genome is made of LD blocks (116). In each block, the SNPs are in tight LD, and certain haplotypes are preferred. The average length of the LD blocks is 7–16 Kb, and on average the LD blocks contain 30–70 SNPs. Because each SNP has two alleles, there are 2^{30} to 2^{70} possible combinations or haplotypes. Amazingly, in all four populations tested, an average of four to six haplotypes were observed in each block, demonstrating the tight LD in each block (116). The significance of the HapMap project is that it now allows us to identify complex disease genes using tag SNPs (Fig. 2). Tag SNPs are representative SNPs from LD blocks. If a tag SNP shows association with disease, it indicates that the gene predisposing to the disease is most likely located within the same block as the tag SNP. This makes fine-mapping of linked regions much easier. Moreover, tag SNPs can now be used to screen the entire human genome. To screen the entire human genome, ap-

A		
	B1 (q1)	B2 (q2)
A1 (p1)	p1q1	p1q2
A2 (p2)	p2q1	p2q2

B		
	B1 (0.2)	B2 (0.8)
A1 (0.3)	0.06 (0.14)	0.24 (0.16)
A2 (0.7)	0.14 (0.06)	0.56 (0.64)

FIG. 1. The principle of LD. A, Assuming two SNPs, A and B, each with two alleles (A1/A2 and B1/B2, respectively) with allele population frequencies of p1/p2 and q1/q2, respectively, the table shows the expected frequencies of each combination of alleles of the two SNPs. For example, the expected frequency of the A1B2 combination is $p1 \times q2$. Any deviation from these expected frequencies is due to LD. For example, if the observed frequency of the A1B2 combination is significantly higher than the expected frequency ($p1q2$), this means that these two alleles are in LD. B, Numerical example of the principle of LD. Alleles A1/A2 have population frequencies of 0.3/0.7, respectively, and alleles B1/B2 have population frequencies of 0.2/0.8. The expected frequencies of each combination of alleles is shown in gray (for example, the combination A1B1 is expected to have a frequency of $0.3 \times 0.2 = 0.06$). The actual observed frequencies are shown in black within parentheses. As can be seen, these deviate significantly from the expected frequencies. For example, the observed frequency for the A1B1 combination is 0.14, and that is significantly higher than the expected frequency of this combination (0.06). Therefore, the A1 and B1 alleles tend to occur together more frequently than expected by random chance, or are in LD.

proximately 500,000 tag SNPs are necessary to have a dense enough SNP coverage. This creates a problem of multiple testing as 500,000 independent tests are being performed. A simple Bonferroni correction would require a genome-wide significance cutoff P value of 1×10^{-7} (113). However, this approach may miss true positive associations that do not result in such a low P value. Despite these difficulties, several replicated genes have been identified recently using genome-wide association studies, for example in Crohn's disease (117), T1D (118), and type 2 diabetes (119).

E. Suggested algorithms for searching for complex disease genes

Unlike the search for genes causing simple Mendelian disorders, it is still not known what is the best approach to identify susceptibility genes for complex diseases. However, recent advances in linkage and association studies, most notably the completion of the human genome and HapMap projects, made mapping and identification of complex disease genes a reality (109, 114). One can use linkage or association-based approaches to map complex disease genes.

1. Linkage-based gene mapping. Linkage-based gene mapping consists of five steps (120).



FIG. 2. LD blocks and tag-SNPs. The HapMap project demonstrated that most of the human genome is composed of LD blocks that contain SNPs in tight LD. Shown here are two LD blocks separated by hot spots of recombination (large filled circles). Each block in the figure contains 10 SNPs (small empty circles). One can then select one (or more) SNPs from each block (arrows) to represent all the SNPs in the block (tag-SNPs). The tag-SNPs can be used to test for association of each block with disease.

a. Identifying linked loci. This is achieved by whole genome screening using microsatellite markers (usually ~400 markers) or SNPs (usually ~5000–10,000 SNPs).

b. Confirming linked loci. A linked locus should be confirmed by finding evidence for linkage in two independent datasets (90). Confirmed loci most likely harbor susceptibility genes, e.g., human leukocyte antigen (HLA) in T1D (112).

c. Fine mapping confirmed loci. Linked loci can be fine mapped by LD mapping. LD mapping is based on association studies with markers that saturate the region of interest. The marker that shows the strongest association with the disease is probably closest to the disease gene. This method can narrow down the region of interest to a few hundred kilobases (98).

d. Testing genes in the linked region. After the linked region has been fine-mapped, the genes in this region can be analyzed. Sequencing of the genes in the fine-mapped loci will identify SNPs that are then tested for association with the disease. If a certain SNP shows a consistently significant association with the disease, it may be the susceptibility allele in the region, although LD with another disease-causing polymorphism cannot be ruled out.

e. Functional studies. To demonstrate that an associated allele is a true susceptibility allele, it is necessary to show that it affects the function of the gene in a way that increases the risk of developing disease. This provides indirect evidence that it may be the actual susceptibility allele for the disease.

2. Association-based gene mapping

a. Genome-wide association using Tag SNPs. This is achieved by testing approximately 500,000 tag SNPs for association with disease.

b. Confirming associated SNPs. Any SNP showing genome-wide significance ($P < 1 \times 10^{-7}$) has to be replicated in one or more independent datasets.

c. Gene identification. Once a SNP has been replicated, the LD block that it is located in most likely contains the disease susceptibility gene. The known genes in the block can be sequenced or known SNPs in them tested for association with disease.

d. Functional studies. As in linkage-based gene mapping functional studies are necessary to show that the associated gene variant affects the function of the gene in a way that increases the risk of developing disease.

IV. A Brief Overview of Susceptibility Genes in T1D and AITD

A. Susceptibility genes in T1D (summarized in Table 2)

So far, more than 18 putative genes/loci have been identified as possible contributors to genetic susceptibility of T1D. However, only four genes/loci have demonstrated functional effects that underlie their influence on disease susceptibility or protection. Of these, clearly the most significant contribution to the genetic risk to T1D is conferred by the major histocompatibility complex (MHC) region (designated as IDDM1). Two other immune regulatory genes, the CTLA-4 and the protein tyrosine phosphatase non-receptor type 22 (PTPN22), have also been shown to contribute to the etiology of T1D. In addition, one target tissue-specific gene, the insulin gene (INS) variable number of tandem repeats (VNTR) polymorphism (designated IDDM2), is also associated with T1D. Many comprehensive reviews on the genetics of T1D have been published recently (121–128). Here we provide only a brief overview on the genetics of T1D, focusing on those genes that are relevant to the joint susceptibility to T1D and AITD.

1. *The MHC II gene locus (IDDM-1)*. Located on the short arm of chromosome 6 (6p21), the MHC locus, encoding the HLA proteins in humans, is the most important T1D susceptibility gene. This locus has been estimated to contribute approximately 40–50% of the heritable risk for T1D (reviewed in Refs. 121 and 129). HLA class II alleles, mainly DQ and DR, were shown to be the primary HLA alleles contributing to the etiology of T1D (129). However, other HLA classes were also found to be associated with T1D, although the risk they confer is lower than the class II alleles.

T1D is strongly associated with the haplotypes DQ2 (DQB1*0201-DQA1*0501-DRB1*03) and DQ8 (DQB1*0302-DQA1*0301-DRB1*04) in Caucasians (27, 129–132). These high-risk haplotypes confer an absolute risk for T1D of ap-

proximately 5% in the general population. However, the risk increases to about 20% in siblings of patients with T1D who carry these risk haplotypes (27, 132, 133). Although only a minority of subjects that carry the high-risk haplotypes develop disease, HLA class II haplotypes are the most important determinants of overall risk for disease. In addition to the risk haplotypes, protective haplotypes, such as DRB1*1501-DQB1*0602 and DR2-DQA1*0102-DQB1*0602 have also been identified (27, 129, 131, 132, 134). Studies have shown that DQB1*0602 may protect specifically from the generation of anti-islet autoantibodies (135, 136). Further sequencing studies have shown a significant correlation between the amino acid at position 57 of the DQ β chain and resistance or susceptibility to disease. If an aspartic acid residue occupied position 57 in both alleles of that chain, T1D was unlikely to occur. Conversely, its absence provided a marker for susceptibility (137–139). The relative risk of T1D for individuals in whom both alleles are non-Asp has been estimated to be 30 to 107 (129, 137). The presence of arginine at position 52 of the DQ α chain has also been shown to confer increased risk for T1D, which is additive with the increased risk conferred by the absence of aspartic acid at position 57 of the DQ β chain (140).

There is wide variation in T1D prevalence worldwide. These variations could be due to environmental and/or genetic differences between populations in different geographic areas. Indeed, several studies have shown that the distribution of DQ-DR haplotypes among different populations around the world may explain part of the worldwide differences in the incidence of T1D (reviewed in Ref. 129). For example, the absence of DR3 haplotypes in the Japanese population may contribute to an overall lower disease frequency, although an alternate haplotype, DR4/DR9, has been reported to represent a high susceptibility within this ethnic group (141, 142).

HLA class II gene profiles may also vary with age of onset

TABLE 2. Known T1D susceptibility genes

Gene	Location	Polymorphisms	Alleles associated with disease	Alleles associated with protection
MHC	6p21	Class II DQ and DR	DR3, DR4, DQ2 (DQB1*0201-DQA1*0501-DRB1*03) DQ8 (DQB1*0302-DQA1*0301-DRB1*04)	DRB1*1501-DQB1*0602 DR2-DQA1*0102-DQB1*0602
		Class III MICA	MICA5 (childhood onset) MICA5.1 (adult onset/LADA)	
INS	11p15	Class I, Class II, Class III VNTR repeats	Class I	Class III
PTPN22	1p13	rs2476601 (C/T)	T allele changing from Arg to Trp at position 620 (stronger association in females)	
CTLA4	2q33	rs57563726 (A/G49) rs3087243 (CT60) (AT) _n	G allele G allele	A allele T allele 86-bp allele
IL2RA/CD25	10p15	Multiple: rs706778 (A/G) rs3118470 (C/T) rs41295061 (A/C) rs11594656 (A/T)	A allele C allele C allele (C/C genotype) T allele (T/T genotype) adds significantly to the effect of rs41295061	G allele T allele A allele A allele
IFIH1/MDA5	2q24	rs1990760 (A/G)	G allele (G/G and G/A genotype)	A allele (A/A genotype)
FoxP3	Xp11,23		Recessive X-linked mutations cause a rare monogenetic disorder with diabetes phenotype (IPEX)	

LADA, Latent autoimmune diabetes of the adult; IPEX, immunodysregulation polyendocrinopathy enteropathy X-linked.

of disease. Caillat-Zucman *et al.* (143) found that T1D patients older than age 15 at onset showed a significantly higher percentage of non-DR3/non-DR4 genotypes than those with younger onset of T1D. Another study, examining correlations with age, confirmed that subjects presenting in childhood had a stronger association with the DQB1*0302 risk allele than patients presenting at ages older than 20 (135). A more recent study showed that the relative risk for development of T1D for both the high and low risk HLA class II genotypes diminished with increasing age of diagnosis (144). Thus, it seems that the HLA effects on the risk of T1D are much more pronounced in early onset disease than in late onset disease, suggesting that environmental factors may play a greater role in adult onset T1D.

The MHC class I chain related gene (MICA), located within the class III region of the MHC locus, is also associated with T1D (145, 146). Here, again, the association was age related. In patients whose disease onset was at an age less than 25 yr, T1D was associated with the MICA5 allele, whereas in patients who developed disease at an age greater than 25 and in patients with latent autoimmune diabetes of the adult, the association was with the MICA5.1 allele (reviewed in Ref. 145).

2. *The insulin gene (INS)*. The first locus outside the HLA region shown to be associated with T1D was the INS region on chromosome 11p5 (designated IDDM2). This locus was estimated to contribute approximately 10% of the genetic disease susceptibility to T1D (122, 147). The associated locus is a polymorphic region that contains a VNTR within the regulatory region of the INS. The VNTR is located close to a DNA sequence that regulates INS expression. Based upon the number of repeats, the length of the VNTR can be divided into three classes: class I (~570 bp), class II (~1640 bp), and class III (~2400 bp). Homozygosity for the short class I alleles confers a 2- to 5-fold increase in the risk for T1D, whereas class III alleles are protective (reviewed in Ref. 122).

Recent exciting data suggest that the INS VNTR may predispose to T1D by altering the transcription of the INS gene in the thymus, where expression of self-antigens during fetal development induces central tolerance. The presence of class III VNTR alleles was associated with increased levels of insulin mRNA in fetal human thymus, whereas class I alleles caused decreased INS expression in the thymus (148, 149). Decreased INS gene expression in the thymus can prevent the negative selection of INS-specific T lymphocytes, thus facilitating escape from central immune tolerance to INS and development of T1D (reviewed in Ref. 122).

3. *The protein tyrosine phosphatase non-receptor type 22 gene (PTPN22)*. (For a detailed discussion of the PTPN22 gene see Section V.C.) The lymphoid tyrosine phosphatase (LYP), encoded by the PTPN22 gene, is a powerful inhibitor of the T cell receptor signaling pathway (150). A coding SNP at nucleotide 1858 of PTPN22 that causes an arginine to tryptophan substitution at position 620 (R620W) was found to be strongly associated with T1D, making it the third most significant T1D gene after the HLA and VNTR loci (151–155).

4. *The cytotoxic T lymphocyte associated protein 4 (CTLA-4) gene*. (For a detailed discussion of the CTLA-4 gene, see Section

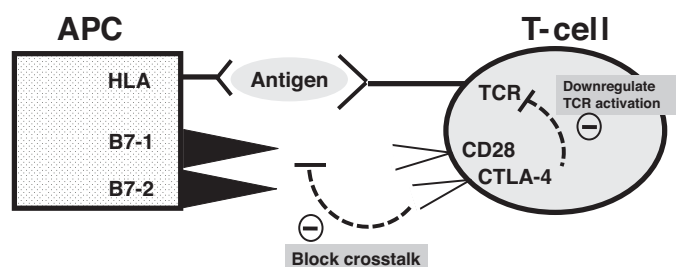


FIG. 3. CTLA-4 function. APCs present peptide antigens to T cells within peptide pockets of HLA class II molecules. However, to activate T cells, costimulation is required. One costimulatory molecule is CD28, which is activated by B7-1 and B7-2 molecules on the surface of APCs. CTLA-4 suppresses T cell activation either by competing with CD28 for binding to B7-1 and B7-2 or by direct suppression of T cell receptor (TCR) signaling pathway.

V.B.) The CTLA-4 on chromosome 2q33 is a major negative regulator of T cell-mediated immune responses (Fig. 3) (156). CTLA-4 was found to be associated with several autoimmune diseases (reviewed in Ref. 157). However, studies in T1D have been mixed (158, 159), with some reporting an association of T1D with CTLA-4 (160–162) and others showing no association (163, 164). We and others have recently shown that the most likely reason for these discrepant results is that the CTLA-4 gene plays a role only in a subset of T1D patients who also develop thyroid autoimmunity (see Section V.B.).

5. *Genome scans in T1D*. Both linkage and association genome scans have been performed in T1D, revealing numerous potential additional loci that contribute to the etiology of T1D. Concannon *et al.* (165) reported whole genome linkage analysis of four datasets, including three previously published scans and one new genome scan totaling 1435 families with 1636 sib-pairs. In addition to the HLA region, nine non-HLA loci showed evidence of linkage to T1D, including loci on 5q, 6q21, and Xp, regions that have been reported to be linked with other autoimmune diseases (165, 166).

More recently, robust genome-wide association studies have been performed in T1D. One of the largest genome-wide association studies in T1D, the Wellcome Trust Case Control Consortium study, confirmed the known associations with the MHC class II locus, as well as the INS, CTLA-4, and PTPN22 genes (118). At least two additional gene loci have been identified, the IL-2 receptor α chain (IL2RA, also known as CD25) gene on 10p15 and the IFIH1 gene (also known as MDA5) on 2q24 (118). In addition, 10 more presumed loci have been identified, but further confirmation is needed. Taken together, these data demonstrate that to a large extent the genetic contribution to T1D involves several major genes (HLA class II, INS VNTR, PTPN22), as well as many other genes with small effects. These genes most likely interact with each other, as well as with environmental factors.

6. *Gene-gene interactions*. Bjornvold *et al.* (144) recently published a comprehensive analysis of gene-gene interactions in T1D, analyzing the four established T1D genes (MHC class II, insulin, PTPN22, and CTLA-4). They used both a case-control cohort and a family trio dataset. Not surprisingly,

they reported that the more susceptibility risk alleles an individual carries, the higher the relative risk of developing disease. All interactions, except between HLA and PTPN22, fitted a multiplicative model, and when all risk alleles were included in the analysis, the resulting odds ratio was 61 (144). Nevertheless, the authors assert that the presence or absence of HLA remains the most important single contributor to the overall risk. Additional gene interactions are likely to confer either protection or susceptibility. However, only a small proportion of the population (and of T1D patients) simultaneously carry HLA and multiple non-HLA susceptibility genotypes, and therefore, determination of an absolute risk of joint susceptibility is imprecise (168).

B. Susceptibility genes in AITD

In the past decade, significant progress has been made in our understanding of the genetic contribution to the etiology of AITD. It is exciting that several AITD susceptibility genes have been identified and characterized. Some of these susceptibility genes are specific to either GD or HT, whereas others confer susceptibility to both conditions.

1. *HLA-DR*. HLA-DR3 was the first confirmed gene to be associated with GD (reviewed in Ref. 169). The frequency of DR3 in GD patients is about 40–50%, and in the general population it is about 15–30%, resulting in an odds ratio of up to 4.0 (170). Among Caucasians, HLA-DQA1*0501 was also shown to be associated with GD [relative risk (RR) = 3.8] (171, 172), but it appears that the primary susceptibility allele in GD is indeed HLA-DR3 (HLA-DRB1*03) (173, 174).

The exact amino acid sequence in the DRβ1 chain conferring susceptibility to GD was unknown until recently. Therefore, we have sequenced the HLA-DRB1 locus in a population of GD patients and controls, and we identified arginine at position 74 of the HLA-DRβ1 chain (DRβ-Arg-74) as the critical DR amino acid conferring susceptibility to GD (174). These data were replicated in an independent dataset (175). Further analysis has shown that the presence of glutamine at position 74 was protective for GD (174). This suggests that position 74 of the DRβ1 chain is critical for GD development.

Data on HLA haplotypes have been less definitive in HT than in GD. Earlier studies showed an association of goitrous HT with HLA-DR5 (RR = 3.1) (176) and of atrophic HT with DR3 (RR = 5.1) in Caucasians (177). Later studies in Caucasians reported weak associations of HT with HLA-DR3 (178, 179) and HLA-DR4 (180).

2. *CD40*. CD40 is a member of the TNF receptor family of molecules that is expressed primarily on B cells and other antigen-presenting cells (APCs) (181). CD40 plays a fundamental role in B cell activation, inducing B cell proliferation, Ig class switching, and antibody secretion (182, 183).

Recently, we and others have identified CD40 as a susceptibility gene for GD (reviewed in Ref. 184). We identified a new C/T polymorphism, at the 5'UTR of CD40, with the CC genotype of this SNP showing significant association with GD (185). With the exception of a sole report (186), the association between the CC genotype of the CD40 5'UTR SNP and GD has now been replicated in several studies,

performed in different populations including Caucasians, Koreans, and Japanese (reviewed in Ref. 184).

The CD40 SNP resides in the Kozak sequence of the 5'UTR of CD40, a region that is essential to the start of translation (187), and thus it was possible that the 5'UTR SNP influenced the translation of CD40. Indeed, functional analysis has shown that the C-allele of the 5'UTR SNP increases the translation of CD40 mRNA transcripts, by 20–30% compared with the T-allele (188, 189). At least two potential mechanisms can explain how the C-allele of the CD40 5'UTR SNP increases the risk for GD: 1) the C-allele may increase CD40 expression and function on B cells, thereby potentially lowering the threshold of activation of thyroid autoreactive B cells; and 2) the C-allele may increase the expression of CD40 in the thyroid gland itself (190, 191). Because thyroid cells have been shown to act as APCs under certain conditions (192), overexpression of CD40 on thyrocytes could enhance the co-stimulation of T cells by thyrocytes (193). These two putative mechanisms are not mutually exclusive, and both may be operating in GD.

3. *CTLA-4*. Since the first report by DeGroot and colleagues (194) in 1995, the CTLA-4 gene was shown in numerous studies to be a major AITD susceptibility gene (158, 161, 162, 195–197). The association between AITD and CTLA-4 has been consistent across populations of different ethnic backgrounds (194, 195, 198–203).

Several CTLA-4 variants are associated with AITD. Three CTLA-4 variants have shown the most consistent associations with AITD, including an (AT)_n microsatellite within the 3'UTR region of the CTLA-4 gene (194, 195), a SNP at position 49 in the CTLA-4 leader peptide (designated A/G₄₉), resulting in an alanine/threonine substitution (60, 162, 202, 204, 205), and a SNP (designated CT60) located near the 3'UTR of the CTLA-4 gene (161).

Interestingly the CTLA-4 gene also confers susceptibility to the production of TAb without clinical disease (206–208), thus substantiating its role as a general autoimmunity gene (see Section V.B).

4. *PTPN22*. The PTPN22 R620W variant is associated with both GD (209) and HT (210). However, unlike CTLA-4, which was associated with AITD across ethnic groups, the PTPN22 gene shows significant ethnic differences in associations. In fact, the susceptible tryptophan variant is not seen in the Japanese population (211). This could be due to founder effects, *i.e.*, the founders of the PTPN22 R620W variant may have been Caucasians, and therefore the variant is rare in the Japanese population.

5. *The Tg gene*. Tg represents one of the major targets of the immune response in AITD (212) and in experimental autoimmune thyroiditis (213, 214). Recently, the Tg gene was established as a major AITD susceptibility gene (98, 215, 216). Sequencing of the entire Tg coding sequence revealed four new SNPs that were significantly associated with AITD (115). All the associated Tg SNPs (except one) were nonsynonymous SNPs, *i.e.*, they caused amino acid changes in the Tg protein. The association between Tg and AITD has been replicated in several other datasets (217–219), although the associated Tg polymorphism might be different in different

populations (218, 220). Several mechanisms can be postulated to explain the association between Tg amino acid variants and AITD. Tg variants could predispose to thyroid autoimmunity by 1) influencing Tg peptide repertoire formed during Tg processing by APCs in endosomes; and/or 2) enhancing binding of Tg peptides to MHC class II molecules (possible Arg-74 containing DR pockets), thereby facilitating the activation of T cells by Tg peptides. Supporting the second mechanism are recent findings that one of the Tg SNPs interacts with the Arg74 variant of HLA-DR, resulting in a high odds ratio of 15 for GD (221).

6. *The TPO gene.* Jaume *et al.* (222) reported that autoantibodies recognizing immunodominant epitopes of TPO are genetically transmitted within families. This genetic transmission of TPO antibody epitopes could be caused by inherited variants in the TPO gene. However, two studies showed no evidence of linkage and/or association of the TPO gene with AITD (223, 224). Hence, it seems that the TPO gene is not involved in the genetic susceptibility to AITD.

7. *The TSH receptor (TSHR) gene.* The hallmark of GD is the presence of stimulating TSHR autoantibodies. Not surprisingly, the TSHR gene was long thought to be an obvious candidate gene for GD (reviewed in Ref. 184). Earlier studies examining SNPs in the extracellular domain of the TSHR for association with GD gave inconsistent results (199, 203, 225–231). More recently, consistent associations between the TSHR gene and GD were reported. Intriguingly, all the associated TSHR SNPs are intronic (232–234). It remains to be determined how the intronic SNPs in the TSHR gene could predispose to GD, but one attractive mechanism is by influencing the splicing of the TSHR gene.

V. Joint Susceptibility Genes for T1D and AITD

Several genes have been shown to contribute to the joint susceptibility to T1D and AITD. Not unexpectedly, most of them are involved in immune regulation.

A. The HLA class II gene locus

As mentioned earlier, both T1D and AITD are associated with the HLA class II region. Therefore, HLA class II is an obvious candidate gene-locus for the joint susceptibility to T1D and AITD. Indeed, with the exception of a few reports (82, 235–237), all studies showed a strong effect of HLA class II genes on the co-occurrence of T1D and AITD within families and in the same individual (APS3v). Moreover, this association was consistent across different ethnic groups, including Caucasians (73, 78, 83, 91, 111, 238–241), Japanese (242, 243), Koreans (244), and Chinese (245). Thus, it is clear that HLA class II genes play a major role in the genetic association between T1D and AITD.

1. *Family studies.* In these studies, cohorts of families in which T1D and AITD clustered were analyzed for cosegregation of HLA class II alleles with both T1D and AITD (Table 3). Our group performed two linkage and association studies in families in which both T1D and AITD clustered (91, 111). In our first study (111), we analyzed a group of 40 multiplex families and found evidence for linkage of the HLA region to T1D (MLS = 7.3), to HT (MLS = 1.5), and to both (MLS = 3.8). Our results suggested that when AITD clusters in families together with T1D, the inheritance of the disease is much more influenced by HLA than when AITD is not associated with T1D. Moreover, the TDT analysis revealed significant association of both T1D and AITD with HLA-DR3; however, only T1D was associated with HLA-DR4. In our second study, we have expanded our dataset to 55 multiplex families, and we have examined the contribution of the HLA-DQ locus in addition to the HLA-DR locus (91). Linkage analysis of the HLA locus confirmed again the evidence for linkage of the HLA locus to T1D, AITD, and both. The TDT analysis revealed preferential transmission of HLA haplotypes DR3-DQB1*0201 and DR4-DQB1*0302 to offspring affected with T1D alone as well as to offspring affected with both T1D and AITD (APS3v). However, when looking at offspring affected with AITD alone, only DR3-DQB1*0201 was preferentially transmitted. These data suggested that DR3-DQB1*0201 hap-

TABLE 3. Selected HLA studies in families with T1D and AITD

First author, year (Ref.)	Country	Study population	n	HLA alleles/ haplotype	OR, P value, or % of subjects positive for allele
Payami, 1989 (238)	United States	Patients with both T1D and AITD	12 subjects	DR4	$P < 0.001$
Santamaria, 1994 (239)	United States	Patients with both T1D and AITD	39 subjects	DR3 DQB1*0201	$P = 0.0005$
Torfs, 1986 (240)	United States	Families with T1D, AITD, and RA	16 families	DQB1*0302 DR3	$P = 0.03$ 44%
Dorman, 1997 (83)	United States	T1D probands and first-degree relatives with AITD	25 families	DR4 DQA1*0501-DQB1*0201	39% OR = 2.2
Golden, 2005 (91); Levin, 2004 (111)	North America	Families in which T1D and AITD cluster	55 families	DR3-DQB1*0201 DR4-DQB1*0302	$P = 0.0002$ $P = 0.0006$

OR, Odds ratio; RA, rheumatoid arthritis.

lototype confers susceptibility to both diseases, whereas the haplotype DR4-DQB1*0302 is specific to T1D. To dissect whether the DR3 or the DQB1*0201 was the primary allele within the DR3-DQB1*0201 haplotype conferring susceptibility to T1D and AITD, we tested haplotypes containing one, but not both, of these constituent alleles. The analysis has shown that DR3 was the primary allele conferring most of the risk to both T1D and AITD, whereas DQB1*0201, in LD with DR3, may have a secondary role (91).

Other groups also reported that the DR3-DQB1*0201 is the critical HLA haplotype conferring joint susceptibility to T1D and AITD. Santamaria *et al.* (239) compared 39 subjects with both T1D and AITD to 17 AITD-only affected siblings of T1D probands. They showed that individuals with both T1D and AITD were more likely to have alleles DQB1*0201 and DQB1*0302, whereas individuals with AITD only were more likely to have DQB1*0201 but not DQB1*0302 (239). One earlier study, examining HLA alleles in families whose members had both T1D and AITD, reported association with DR4 in addition to DR3 (238). Dorman *et al.* (83) studied 25 T1D families in which at least one parent and one offspring had HT and found a 2-fold increase in the prevalence of DQA1*0501-DQB1*0201 among family members with HT compared with those without. No difference in the prevalence of DQA1*0301-DQB1*0302 among these two groups was observed. In summary, although it is possible that other genes in LD with HLA-DR3 contribute to the joint susceptibility to T1D and AITD, most of the data support the DR3-DQB1*0201 as the primary HLA haplotype contributing to the clustering of T1D and AITD within families.

2. Population studies. Here the approach is to compare the frequency of HLA class II alleles/haplotypes in patients with

T1D alone *vs.* patients with T1D and AITD (Table 4). Holl *et al.* (73) looked for correlations between HLA class II alleles and the prevalence of TAb in a population of 495 diabetic children in Germany. Patients carrying the DR3/DR4 genotype had a higher prevalence of thyroid autoimmunity compared with patients carrying other DR genotypes (DR3/DRX, DR4/DRX, and DRX/DR-X, where X stands for non-DR3, non-DR4 alleles). This analysis suggested that both DR3 and DR4 are associated with thyroid autoimmunity among T1D patients (73). These data are consistent with our data showing that the haplotypes DR3-DQB1*0201 and DR4-DQB1*0302 predispose to the combined phenotype of T1D and AITD in the same individual (APS3v) (91). Two other studies (78, 241) investigated HLA associations in Caucasian patients with variants of APS. Huang *et al.* (241) performed HLA typing on patients with APS2 [autoimmune adrenalitis plus at least one other autoimmune disorder (1)]. The patients were divided into those with evidence of islet cell autoimmunity (clinical T1D and/or ICA or glutamic acid decarboxylase antibodies) and those without islet cell autoimmunity. In the former group, the haplotypes DR3-DQB1*0201 and DR4-DQB1*0302 were more frequent compared with controls. However, in the APS2 patients lacking islet cell autoimmunity, only the haplotype DR3-DQB1*0201 was increased, lending further evidence to the notion that DR3-DQB1*0201 is associated with autoimmunity in multiple endocrine organs (241). Wallaschofski *et al.* (78) reported slightly different findings. In this study, 112 APS patients were divided into groups of 29 patients with APS2 and 83 patients with APS3 [both as defined above (77)]. Of note, 21 (25%) of the APS3 patients had T1D and 82 (99%) had AITD. The haplotypes DR3-DQB1*0201 and DR4-DQB1*0302 were

TABLE 4. Selected studies showing association of HLA with the co-occurrence of T1D and thyroid autoimmunity.

First author, year (Ref.)	Country	Study population	n	HLA alleles/haplotype	RR or P value
Chikuba, 1995 (242)	Japan	Patients with both T1D and GD	14	DR9	$P < 0.05$
Huang, 1996 (241)	United States	APS type 2 patients with b-cell autoimmunity	17	DQA1*0301 DPB1*0501	$P < 0.05$ $P < 0.05$
				DR3-DQB1*0201	$P < 0.01$
Chuang, 1996 (245)	Taiwan	Patients with T1D and thyroid antibodies	23	DR4-DQB1*0302 DR3-DQB1*0201	$P < 0.01$ $P < 0.05$
				DRB1*0405/DQA1*0301/DQB1*0401	RR = 4.4
Holl, 1999 (73)	Germany	T1D children with thyroid antibodies	NS	DR3/DR4 genotype	$P = 0.08$
Wallaschofski, 2003 (78)	Germany	APS type 2 patients	29	DR3 DR4	$P < 0.001$ $P < 0.05$
				DQA1*0301 DQA1*0501	$P < 0.001$ $P < 0.05$
Kim, 2003 (244)	Korea	Patients with both T1D and thyroid antibodies	18	DR4 DQA1*0301 DQB1*0401	$P < 0.025$ $P < 0.001$ $P = 0.0017$
				DRB1*0405	$P < 0.01$
Hashimoto, 2005 (243)	Japan	Patients with both T1D and AITD	24	DRB1*0802 DQA1*03	$P < 0.0001$ $P < 0.01$
				DQA1*0401	$P < 0.0001$
				DQB1*0401	$P < 0.01$

NS, Not specified.

both increased in the APS2 patients, compared with controls, but only DR4-DQB1*0302 was increased in the APS3 patients. These results are different from those reported in other studies that showed that both haplotypes DR3-DQB1*0201 and DR4-DQB1*0302 contributed to the APS3 variant (APS3v) consisting of T1D and AITD (73, 91, 241, 246). However, Wallaschofski *et al.* (78) did not present subgroup analysis looking specifically at APS3 patients with the T1D and AITD phenotype. Indeed, a recent study from Germany showed again that HLA-DR3 is strongly associated with APS3 patients having T1D and AITD (246).

The HLA locus was also studied in other ethnic groups. One study from Japan reported a higher prevalence of HLA DR9, as well as DQA1*0301 and DPB1*0501, in Japanese diabetics with GD (242). In contrast, Hashimoto *et al.* (243) reported that the frequencies of HLA alleles DRB1*0405, DRB1*0802, DQA1*03, DQA1*0401, and DQB1*0401 were significantly higher in APS3 patients with T1D and AITD, when compared with healthy controls. The DQB1*0401 allele was also reported to be associated in two studies from Korea (244) and Taiwan (245). In the Korean study, the DQB1*0401 allele was found more frequently in patients with both T1D and HT than in healthy controls or nondiabetic AITD patients (244), suggesting that DQB1*0401 is a predisposing genetic marker for the development of HT in patients with T1D, but not in patients without. The Taiwanese study showed that the DRB1*0405/DQA1*0301/DQB1*0401 haplotype was significantly increased in the subgroup of patients with both T1D and AITD, but not in patients with only T1D, again supporting a major role for the DQB1*0401 allele in Asian patients (245). Taken together, these data suggested that different HLA class II alleles predispose to APS3v in different ethnic groups. However, the structural-functional mechanisms underlying these differences have not been investigated thus far.

B. CTLA-4

1. *CTLA-4 and autoimmunity.* CTLA-4 polymorphisms have been shown to be associated with a variety of autoimmune conditions (reviewed in Refs. 157, 184, and 247), including asthma (248), Addison's disease (201), myasthenia gravis (249), Sjogren's syndrome (250), systemic lupus erythematosus (SLE) (251), systemic sclerosis (252), and ulcerative colitis (253). However, by far the most consistent association reported is with AITD. All forms of AITD, including GD, HT, and the presence of TAb, have been shown to be associated and linked with the CTLA-4 gene/locus (184, 254). In contrast, analysis of the CTLA-4 gene in T1D gave inconsistent results (158–164), with some studies showing association of T1D with CTLA-4 (160, 162) and other studies showing no association (163, 164). A recent very large study showed association of T1D with CTLA-4, but the RR was very low (1.1) (161). We hypothesized that the weak effect of CTLA-4 in T1D may be secondary to a subset effect (for detailed discussion of subset effects, see *Section VII*). Therefore, we focused on the subset of T1D patients that also have thyroid autoimmunity (APS3v patients).

2. *CTLA-4 in T1D and AITD (APS3v).* To test the hypothesis that CTLA-4 is linked only with the subset of T1D patients

that also have AITD, we analyzed the CTLA-4 locus in a dataset of families in which both T1D and AITD clustered. Although no significant LOD score was seen when analyzing all T1D patients as affected, a significant LOD score was obtained when considering only patients with T1D and AITD (APS3v) as affected. Thus, our data showed that the CTLA-4 gene was linked only in the subset of patients that had both T1D and AITD. These data were in keeping with two other studies that examined the CTLA-4 gene in APS patients. Kemp *et al.* (255) found an association of CTLA-4 with an APS variant in which the main components were vitiligo with AITD or T1D. Another study from Japan found an association between the G allele of the CTLA-4 A/G49 SNP with T1D in younger patients that also had AITD (256). Two more recent studies also confirmed our data. Howson *et al.* (92) studied the CTLA-4 gene in a large cohort (>4000) of T1D patients and divided them into those that had positive TPO antibodies and those that were negative for TPO antibodies. Although the odds ratio for the TPO-negative subset for association with the CTLA-4 rs3087243 (CT60) SNP was only 1.16, the odds ratio in the subset of TPO antibody-positive T1D patients was 1.49 (92). These data support the notion that the subset of T1D patients that develop thyroid autoimmunity (APS3v) is the subset most influenced by the CTLA-4 gene. Similarly, Ikegami *et al.* (93) reported that the CTLA-4 A/G49 SNP was associated only in the subset of T1D patients that also had AITD. Taken together, these data demonstrate that the CTLA-4 gene contributes to the expression of APS3v but does not contribute (or has minimal contribution) to the susceptibility to T1D alone (91).

C. PTPN22

1. *PTPN22 and autoimmunity.* The allelic variation of the PTPN22 gene, R620W, was found associated with several autoimmune disorders (257), including rheumatoid arthritis (258, 259), SLE (260–262), vitiligo (153), T1D (151), and AITD (184). This suggested that PTPN22 may increase immune responsiveness, thereby predisposing to autoimmunity in general. However, some autoimmune diseases, such as multiple sclerosis (210) and Addison's disease (154), did not show an association with PTPN22. This could be due to interaction (or lack thereof) between PTPN22 and other genes and/or environmental factors.

2. *PTPN22 is associated with both T1D and AITD.* The association between a PTPN22 R620W polymorphism and T1D was first reported by Bottini *et al.* (151) in two series of patients with T1D from the United States and Sardinia. The odds ratio for T1D was approximately 2 (151). Smyth *et al.* (263) replicated this association in 1388 diabetic families and 1599 isolated diabetic subjects from the United Kingdom, the United States, and Romania with a similar odds ratio of 1.8. These data have now been confirmed in case-control as well as in family-based studies performed in several Caucasian populations, from the United States (264–266), Canada (267), Italy (152, 268), and the Netherlands (153, 269), as well as in U.S. Latinos (270).

Interestingly, some studies performed in Caucasians have suggested a gender preference for the association of PTPN22

with T1D. Two recent studies reported that the R620W variant is associated with T1D only in females, not in males (154, 271). Furthermore, the TT genotype was associated with younger age of onset of T1D compared with the TC and CC genotype (271). Another subset that has been investigated is T1D with young age of onset. Two studies, performed in Spanish (155) and Danish (272) populations, have reported that the R620W variant was associated in the subset of female T1D patients with young age of onset of T1D. These studies demonstrate the power of subset analysis (see *Section VII.B*).

The PTPN22 R620W variant was reported to be associated with both GD (209, 263, 273, 274), and HT (210), although the association with HT has not been as consistent (154) as with GD. One study found an association with young age of onset of GD, again demonstrating the importance of subsetting phenotypes by clinical and demographic parameters (273).

Studies in different ethnic groups demonstrated that, unlike CTLA-4, PTPN22 shows significant ethnic differences in the association with AITD, most probably due to founder effects and/or due to the absence of susceptible variants in certain ethnic groups. Ban *et al.* (211) did not find an association of R620W with AITD in a Japanese population; in fact, none of the patients and the controls had the tryptophan (W) allele. Their data were replicated by Mori *et al.* (275). The R620W is also not polymorphic in Koreans (276) and is rare in African-Americans (275). Thus, unlike CTLA-4 polymorphisms, the PTPN22 R620W variant seems to be specific for Caucasians. However, sequencing of the entire PTPN22 gene demonstrated additional SNPs that showed weak associations with T1D in Japanese and Korean patients (276).

3. *Analysis of the PTPN22 gene in APS3v (T1D and AITD)*. It is clear that, at least in Caucasians, the PTPN22 gene plays a key role in the genetic susceptibility to several organ-specific autoimmune diseases (257). To date, only one study has tested the PTPN22 gene in patients with both T1D and AITD (APS3v) (268). Saccucci *et al.* (268) first replicated the association between T1D and the PTPN22 R620W variant in two populations of Italian diabetic patients. They then tested whether the W allele was associated with a subset of T1D patients affected by another autoimmune disease. They found an association with HT, but not with celiac disease, in one of the populations (268). In addition, two other studies analyzed the R620W polymorphism of PTPN22 in subgroups of patients with multiple autoimmune diseases (210, 261). Wu *et al.* (261), in a family-based association study, found an increased frequency of the W allele among 54 SLE patients with AITD when compared with 601 individuals with SLE alone. In another family-based study, Criswell *et al.* (210) showed that the R620W polymorphism confers risk of T1D, rheumatoid arthritis, SLE, and HT in 265 families with multiple autoimmune phenotypes. Thus, the strong association of PTPN22 with both T1D and GD, as well as studies in families with multiple autoimmune diseases, suggests that it likely plays a role in the joint susceptibility to T1D and AITD. However, this still awaits confirmation.

D. Other potential genes

Several additional genes have shown suggestive associations with T1D and AITD. They are summarized in Table 5. These still await confirmation.

TABLE 5. Known and putative joint susceptibility genes for T1D and AITD

	SNP or variant associated	Ref.	
		AITD papers	T1D papers
Genes successfully confirmed for APS3v			
HLA class II genes	6p21	174, 175, 343, 344, 91, 178, 180	345
CTLA-4	2q33	162, 194, 202, 227, 346–348, 161	158, 161
PTPN22	1p13	209, 210, 263, 274	151, 263
Putative genes for APS3v, associated with both AITD and T1D			
CD25	20 tagged SNPs	349	350
Insulin gene (INS, IDDM2)	11p15	351	352–354
TNF- α	–308 G/A promoter	355–358	359–361
	–238 G/A promoter		
	–863 C/A promoter		
PDCD-1 (PD-1)	+7146 G/A	362	363, 364
	–606 G/A		
	+91 C/T		
	+202 G/c		
	+317/+318 non-ins/ins		
	+6371 G/A		
	+7558 C/T		
	+7718 T/C		
IFIH1	rs1990760 (A/G)	365	366
Possible genes with inconsistent results			
IL-4/IL-4R	–590 C/T promoter and others	367–370	371–375
Vitamin D receptor	C/T (FokI)	376–380	371
IL-13	–1512 A/C SNP	381, 382	372, 375
FOXP3	(GT)(n) and (TC)(n) microsatellites	278, 383	279–281
X-chromosome various loci	Xp	98, 167, 384–386	277

VI. Whole Genome Linkage Analysis in Families with T1D and AITD

So far only three genes have been confirmed to confer joint susceptibility to T1D and AITD (see *Table 5*) (166). However, other genes must exist. To identify these additional genes, linkage studies in families in which both T1D and AITD cluster (“T1D-AITD families”) would be ideal because these genes will be highly penetrant in such families (82). However, until recently no whole genome linkage studies were performed in T1D-AITD families. Recently, we have completed the first whole genome scan in a large cohort of multiplex T1D-AITD families (277). The T1D-AITD families in our dataset were analyzed for linkage under two models. In model 1, individuals with T1D or AITD (or both) were considered as affected; a locus showing linkage under this model contributes to the clustering of T1D and AITD within the same family. In model 2, only individuals with both T1D and AITD (APS3v) were considered as affected. Loci identified using this model contribute only to the combined phenotype of T1D and AITD in the same individual (APS3v).

A. Loci linked with T1D or AITD

When we analyzed our families for linkage with 400 markers spanning the entire human genome using this model, three loci were identified on chromosomes 2, 6, and X. The chromosome 6 locus marker is located within the HLA class II gene locus, and this locus most likely represents linkage with HLA class II. The chromosome X marker is close to the forkhead box P3 (FOXP3) gene, which was previously reported to be associated with AITD (278) and T1D (279), although the data are inconsistent (280) with some reports showing no association with T1D (281). At present, we cannot determine whether the FOXP3 gene itself or another gene in this locus is the susceptibility gene for T1D and AITD. Additional fine-mapping studies are required. In summary, our linkage analysis for loci that contribute to the clustering of T1D and AITD in families identified one previously reported locus, HLA DR/DQ, and two new loci on chromosomes 2 and X. These loci are currently being fine-mapped.

B. Loci linked with T1D and AITD (APS3 variant)

Next we analyzed our families considering only individuals with the APS3v (those that had both T1D and AITD) as affected. Under this model our whole genome linkage analysis identified three loci showing evidence for linkage. The first locus was the CTLA-4 gene locus, thus, extending our previous data obtained in a smaller dataset of families (91). A second locus, on chromosome 6, is most likely the HLA class II gene locus, again extending our previous results (91, 111). Thus, our data confirmed that CTLA-4 and HLA class II are major genes for APS3v.

Interestingly, the third locus was again on chromosome X in the FOXP3 gene region. Thus the FOXP3 gene region most likely contains a major gene predisposing to both T1D and AITD. An APS3v susceptibility gene on chromosome X would be expected to cause a female preponderance of this phenotype because females inherit two X-chromosomes from their parents (compared with one in males) and are,

therefore, more likely to inherit an X-chromosome gene than males. Indeed, in our own dataset the female-to-male ratio of the APS3v phenotype was 1.8:1 (277). It remains to be determined which gene in this region is the susceptibility gene for APS3v.

VII. From Association Studies to Gene Function: Emerging Mechanisms of Joint Susceptibility to T1D and AITD

A. General principles

In classical monogenic diseases, one or more mutations either inactivate a gene [*e.g.*, the autoimmune regulator gene in APS1 (282)] or cause unchecked activation of the gene [*e.g.*, the RET protooncogene in multiple endocrine neoplasia type 2 (MEN2) (283, 284)]. However, in complex diseases such as T1D and AITD, the genetic defect may cause subtle changes in the function of one or more genes. These small changes in the function of several genes, when combined, increase the likelihood to develop the disease. Therefore, even when a gene causing a complex disease is mapped, proving that a certain variant changes the function of the gene in a way that will promote the development of the disease can be challenging. However, a gene variant can be declared causative only after it is demonstrated that it alters the gene function in a way that will increase the risk for disease.

B. The crucial role of subset analysis

As new genes/loci are being discovered for complex diseases, it is becoming evident that singly most have a weak association with disease. In the case of T1D and AITD, with the exception of the HLA class II genes, most susceptibility genes identified so far increase the risk for disease by a modest odds ratio of 1.2–2.0. How can we reconcile the abundant epidemiological evidence for a strong genetic predisposition for T1D and AITD with the relatively weak effects of the genes identified? One favored explanation is that it is the combination of many small effects that determines the final risk. The risk may be further increased by gene-gene interactions. Indeed, there is evidence, including from our group, for interactions between susceptibility genes (221). However, the chances of inheriting multiple susceptibility alleles are low, and it is not likely that an individual will inherit more than a few interacting alleles. Therefore, other mechanisms must explain the low odds ratios observed for most susceptibility genes for T1D and AITD. One attractive mechanism is that certain susceptibility genes give a strong risk for disease only in a subset of patients; therefore, when testing all patients, the effect on the subset gets “diluted” and the odds ratio decreases (285). In Fig. 4 we demonstrate the effect of diluting the subset that is associated with a disease gene with the whole set of patients. As can be seen under certain assumptions, even diluting the subset to 60% of the total dataset will already result in no association or very weak association with a gene variant. Indeed, a subset analysis performed by us in a dataset of AITD families identified subset-specific loci that were previously not identified (286). Therefore, identifying, subsets of patients likely to be influ-

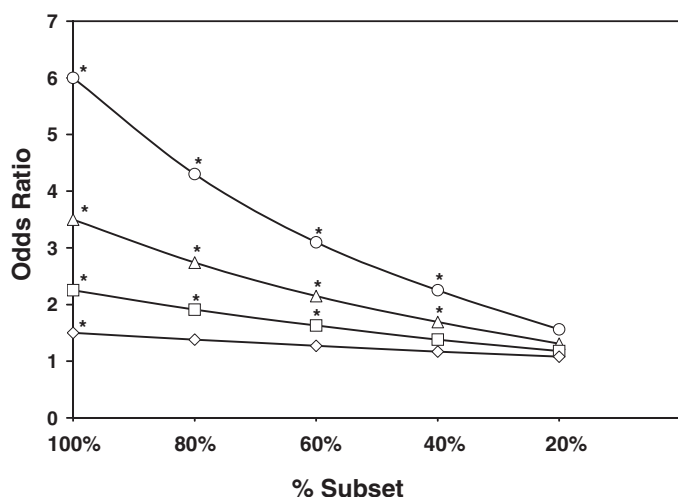


FIG. 4. A graph demonstrating the effect of subset size on the odds ratio of an association with a certain gene variant. The x-axis shows the percentage of patients among the entire dataset that belong to the subset that is associated with the gene variant. The y-axis shows the odds ratio. Simulations were made assuming a dataset of 200 patients and 200 controls. We also assumed that the frequency of the disease-associated variant is 60% in the subset of patients that is associated with this gene variant. We then simulated the frequency of the disease-associated variant in the controls and in the patients not belonging to the subset to be 20% (circles), 30% (triangles), 40% (squares), and 50% (diamonds). Asterisks indicate the conditions in which the association is still statistically significant ($P < 0.05$). These simulations show that as the percentage of patients among the entire dataset that belong to the subset decreases, the odds ratio of the association with the gene variant decreases exponentially. For example, assuming a frequency of the disease-associated allele of 40% among controls and patients that do not belong to the subset (squares), the odds ratio drops from 2.3 to 1.2, and once the subset consists of no more than 40% of the entire dataset, the association becomes not significant.

enced by a narrower set of susceptibility genes is key to identifying genetic influences in complex diseases. This is especially important when extrapolating the statistical data into functional data. For example, a gene with a weak effect in a set of patients (*i.e.*, low odds ratio) might mistakenly be thought to have a weak or insignificant biological effect on the development of disease. In fact, if this gene gives a high odds ratio in a subset of patients, then it actually has a strong biological effect, but only in the subset. The importance of subset analysis was recently demonstrated in the association of CTLA-4 and T1D (91, 92). The subset of T1D patients that also develop thyroid autoimmunity is now emerging as a distinct genetic subset, and therefore we have designated it APS3 variant (APS3v).

C. HLA

Over the past three decades, the mechanisms by which HLA proteins confer susceptibility to autoimmunity have been dissected. T Cells recognize and respond to an antigen by interacting with a complex between an antigenic peptide and an HLA class II molecule (mostly DR and DQ) (reviewed in Ref. 287). The various HLA class II alleles have different affinities for peptides. Thus, peptides formed from proteolysis of autoantigens (*e.g.*, islet cell antigens) are recognized

by T cell receptors on cells that have escaped tolerance. These will have differing affinities for different HLA class II alleles (288). Certain alleles may permit the autoantigenic peptide to fit into the antigen binding groove inside the HLA molecule and to be recognized by the T cell receptor, whereas others may not (289). This could determine whether an autoimmune response to that antigen will develop.

The best-studied disease for structural-functional correlations between HLA class II pocket variants and peptide binding is T1D (122). As mentioned before, it was established that the amino acid residue at position 57 of the DQ β chain plays a key role in the genetic susceptibility to T1D (139). Lack of Asp at this position at both DR alleles is strongly associated with T1D (137). Structural analysis of DQ molecules has shown that lack of Asp57 on the DQ β chain may predispose to T1D by causing significant alterations in the pocket structure (290, 291). Crystal structure of the HLA-DQ molecule demonstrated that when Asp is present at position 57 of the DQ β chain, it forms a salt bridge with the arginine at DQ α 76 making pocket 9 (P9) electrostatically neutral. In contrast, lack of the negatively charged Asp at DQ β 57 makes pocket P9 positive and enables insulin peptides to form a salt bridge with Arg at DQ α 76 (290, 292). Thus, lack of Asp at DQ β 57 will permit immunogenic islet cell peptides (*e.g.*, insulin peptides) to fit into the HLA-DQ peptide binding pocket and to be recognized by the T cell receptor (290, 292). In contrast, the presence of Asp at DQ β 57 will prevent insulin peptides from fitting in the pocket and hence will prevent them from being presented to T cells (289).

Similar genetic-structural studies were performed by us in AITD. As mentioned, we have shown that DR β -Arg74 GD is the critical HLA-DR pocket amino acid associated with GD (174). Position 74 of the DR β chain is located in pocket 4 (P4) of the DR peptide binding cleft. Structural modeling analysis demonstrated that the change at position 74, from the common neutral amino acids (Ala or Gln) to a positively charged hydrophilic amino acid (Arg), significantly modified the three-dimensional structure of the P4 peptide-binding pocket (174). This could alter the peptide binding properties of the pocket favoring peptides which can induce GD (174, 293).

How can the same HLA class II pockets confer joint susceptibility to T1D and AITD if the autoantigenic peptides causing each disease are distinct (islet cell antigens in T1D and thyroid antigens in AITD)? There could be at least two potential explanations for this paradox (Fig. 5). First, the alleles predisposing to T1D and AITD are distinct but in tight LD. Thus, whereas different pocket structures predispose to T1D and AITD, both pocket structures are frequently inherited together because the genes coding for these pockets are in tight LD. In other words, due to the tight LD between the T1D- and AITD-associated alleles, individuals are likely to express both variants on their APCs, and therefore, both the islet cell- and thyroid-derived peptides will fit in the pockets (Fig. 5A). Indeed, the GD-associated allele, DRB1*0301, and one of the major T1D-associated alleles, DQB1*0201, are in tight LD (91, 122). Second, the same pocket variant predisposes to both diseases, but its influence is on anchoring the T cell receptor and not on peptide binding (Fig. 5B). During the

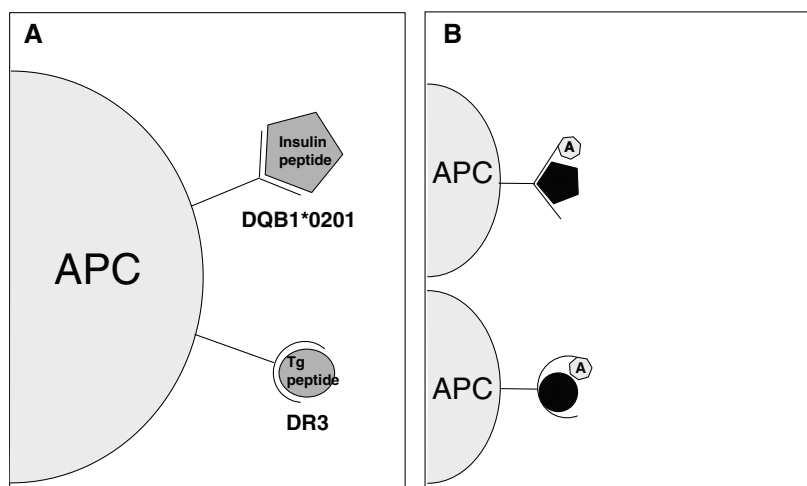


FIG. 5. Two potential mechanisms by which HLA class II molecules can predispose to both T1D and AITD. A, Two distinct HLA class II molecules (e.g., DQB1*0201 and DR3) with distinct pocket structures are frequently inherited together and expressed on APCs because they are in tight LD. B, Two distinct HLA class II molecules with pocket structures fitting different peptides (e.g., insulin and Tg) share an amino acid (marked A) that serves to anchor the HLA class II molecule to the T cell receptor.

presentation of antigenic peptides by HLA class II molecules to the T cell receptor, some of the HLA pocket amino acids serve as anchors for the T cell receptor (294, 295). It is possible that amino acids that show strong association with both T1D and AITD (e.g., arginine at position 74 of the DR β chain) predispose to disease by increasing the contact between the HLA-peptide complex and the T cell receptor. However, at present this hypothesis requires confirmation.

For islet cell or thyroid autoantigens to be presented by HLA molecules to T cells, a mechanism of autoantigen presentation must exist within the target tissue. One potential mechanism not utilizing professional APCs may be through aberrant expression of HLA class II molecules on the target tissue cells (islet cells or thyrocytes) (192, 296, 297). Indeed, there is abundant evidence that, under cytokine stimulation, both islet cells and thyrocytes can be induced to express class II MHC molecules and serve as APCs (298–302). Moreover, thyroid epithelial cells from patients with GD and HT have been shown to express HLA class II antigen molecules similar to those normally expressed on APCs (296, 303, 304). Similarly, there are reports of aberrant expression of HLA class II in islets from patients with T1D (305, 306). This aberrant expression of HLA class II molecules on target tissues may initiate autoimmunity via direct autoantigen presentation by islet or thyroid cells (296, 307) or a secondary event following cytokine secretion by invading T cells. Consistent with the former possibility was the fact that thyroid cell MHC class II antigen expression could be induced by certain viral infections *in vitro* (308, 309) and that mice constitutively expressing human DR3 developed thyroiditis after immunization with human Tg (310). Coculture of peripheral blood mononuclear cells from GD patients with homologous thyrocytes induced T cell activation (311) as well as interferon- γ production and thyroid cell HLA class II antigen expression (312). Such cytokine secretion may be the common cause of HLA class II antigen expression by the target tissues in T1D and AITD (192).

D. CTLA-4

1. *Which CTLA-4 polymorphism is the causative one?* The fact that polymorphisms within the CTLA-4 gene locus show linkage and association with autoimmunity does not necessarily mean that CTLA-4 is the susceptibility gene in this locus. It is possible that another nearby gene in LD with CTLA-4 is the causative gene at this locus. Indeed, the CTLA-4 gene region contains several candidate immune regulatory genes for autoimmunity, such as CD28 and inducible costimulator. Therefore, we (197) and others (161, 313) fine-mapped this region and have shown that the strongest association was with the CTLA-4 gene.

That still left the question of which CTLA-4 polymorphism is the causative one. As mentioned above, several CTLA-4 variants have been associated with autoimmunity. A recent large fine-mapping study using over 100 markers in the CTLA-4 region provided evidence that the causative polymorphism is located in the 3' UTR of the CTLA-4 gene (161). However, several candidate polymorphisms in this 5-Kb region still exist, including the (AT) n microsatellite as well as several SNPs (161). To determine which variant is the causative one, functional studies are needed (see Section VII.D.2).

2. *Functional studies of CTLA-4 in autoimmunity.* The CTLA-4 gene is a 188-amino acid glycoprotein that plays a key role in the interaction between T cells and APCs (reviewed in Ref. 156). APCs activate T cells by presenting to the T cell receptor an antigenic peptide bound to an HLA class II protein on the cell surface. However, a second signal is also required for T cell activation, and these costimulatory signals may be provided by the APCs themselves or other local cells (314). The costimulatory signals are provided by a variety of proteins that are expressed on the cell surface of APCs (e.g., B7-1, B7-2, B7 h, and CD-40) and cross-talk with receptors (e.g., CD28, CTLA-4, and CD-40L) on the surface of CD4 $^{+}$ T-lymphocytes during antigen presentation (314). The complex interaction between APC proteins and T cell proteins during antigen presentation is called the immunological synapse

(Fig. 3). Whereas the binding of B7 to CD28 on T cells co-stimulates T cell activation, CTLA-4 down-regulates T cell activation (315). For example, complexing CTLA-4 with a blocking monoclonal antibody was shown to induce proliferation of T cells and the production of IL-2 (316–318). Additionally, CTLA-4 knockout mice succumb early in life due to uncontrolled proliferation and activation of T lymphocytes that infiltrate and destroy multiple tissues (319–321). CTLA-4 is not constitutively expressed on resting naive CD4⁺CD25[−] T cells (322, 323). Rather, in response to T cell receptor ligation, CTLA-4 expression is induced, peaking 24–48 h later (324, 325). On the other hand, CD4⁺CD25⁺ T regulatory (Treg) cells constitutively express CTLA-4, and therefore, it has been suggested that CTLA-4 plays a role in their function (326). The CTLA-4 molecule initiates its signal in response to its ligation with either the B7-1 or B7-2 proteins expressed on APCs (327, 328).

In view of the function of CTLA-4 as a negative regulator of T cells, one would expect a polymorphism that decreases CTLA-4 function and/or cell surface expression to cause heightened T cell activation and potentially lead to the development of an autoimmune condition (Fig. 3) (184). Several CTLA-4 variants have been analyzed in detail for their effect on CTLA-4 function and/or expression. The A/G49 SNP (rs57563726) causing a Thr to Ala substitution in the signal peptide was reported to cause misprocessing of CTLA-4 in the ER, resulting in less efficient glycosylation and diminished surface expression of CTLA-4 protein (329). However, no further studies have been performed on the effects of this SNP on posttranslational modification of CTLA-4, and this mechanism, while very attractive, awaits confirmation. DeGroot and colleagues (330) examined the effects of the A/G49 alleles on T cell activation. They have shown an association between the G allele of the A/G49 SNP and reduced control of T cell proliferation, results that were later replicated by us (197). This association could be due to a direct effect of the A/G49 SNP or due to the effects of another polymorphism in LD with the A/G49 SNP. To examine these two possible scenarios, Davies and colleagues (331) 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. The results showed no difference in CTLA-4 expression and/or function when the Jurkat cells were transfected with a CTLA-4 construct harboring the A or the G allele (331). Therefore, it was concluded that the A/G49 SNP is, most likely, not the causative SNP, but rather is in LD with the causative variant.

Two promoter SNPs in CTLA-4 have been studied for their effect on function, too. A CTLA-4 −1661 promoter SNP had no influence on promoter activity when either the A or G allele was present (332). On the other hand, analysis of a −318 promoter SNP has shown that the T allele had an 18% higher promoter activity compared with the C allele (333). 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 levels in resting cells (334). However, the −318 promoter SNP association with autoimmunity was weak and not consistent across studies

(335), in contrast to the other CTLA-4 polymorphisms studied, and therefore, the significance of these results is unclear at present.

A recent comprehensive analysis of the CTLA-4 gene locus demonstrated that the CT60 SNP of CTLA-4 (rs3087243) showed the strongest association with GD, suggesting that it might be the causative SNP (161). Further functional analysis in a small number of patients has shown that the GG (disease susceptible) genotype of CT60 was associated with reduced mRNA expression of the soluble form of CTLA-4 (161). However, a recent large study from Sweden could not replicate these results (336), and thus it is unclear whether CT60 is, indeed, the causative variant.

Another attractive CTLA-4 variant that could affect CTLA-4 expression and/or function is the 3'UTR (AT)_n microsatellite. The longer repeats of this microsatellite were shown to be associated with reduced CTLA-4 inhibitory function (337). However, as in the case of the A/G49 SNP, this could be due to LD with another SNP that is the causative one. Further studies have shown that 3'UTR microsatellite affected the half-life of the CTLA-4 mRNA, with long repeats being correlated with shorter half-life compared with the short repeats (338). Indeed, the 3'UTR region of CTLA-4, in which the AT repeats lie, contains three AUUUA motifs that may affect mRNA stability (339). Therefore, this could provide an attractive explanation for the association between the long alleles of the microsatellite and autoimmunity.

Another possible scenario is that no one CTLA-4 variant is causative and that a haplotype consisting of several variants is responsible for the association with autoimmunity. Indeed, it was recently shown that different populations have extended haplotype signatures in the CTLA-4 gene locus (340). Although these signatures were not shown to be associated with disease, this is an attractive possibility.

E. PTPN22

The LYP encoded by the PTPN22 gene is located on chromosome 1p13 and belongs to a family of protein tyrosine phosphatases that are expressed in both immature and mature B and T lymphocytes. LYP is a powerful inhibitor of the T cell antigen receptor signaling pathway (150). LYP binds to the C terminal of the protein kinase, Csk, thus restricting the response to antigens by disrupting protein tyrosine phosphorylation events that control cell activation and differentiation. Subsequently, this negative control mechanism prevents spontaneous T cell activations and reverts activated T cells to a resting phenotype (257, 262).

The exact mechanism by which the R620W variant of the PTPN22 gene predisposes to autoimmunity is not known. However, it has been shown that the substitution of arginine with tryptophan at this position interferes with the interaction of LYP with Csk. *In vitro* experiments show that only LYP with arginine at position 620 forms a complex with Csk, whereas LYP with tryptophan at this position binds less efficiently (153). Intriguingly, the disease-associated tryptophan variant makes the protein an even stronger inhibitor of T cells because it is a gain-of-function change (341). Thus, the diseases-associated tryptophan variant would be expected to suppress T cell activation and proliferation. How can we

reconcile the fact that the tryptophan allele decreases T cell activation with its role in promoting the development of autoimmunity? One possible explanation for this paradox is that a lower T cell receptor signaling would lead to a tendency for self-reactive T cells to escape thymic deletion and thus remain in the periphery. Evidently, further studies are needed to clarify the role of the W620 variant in T cell activation and autoimmunity.

VIII. Conclusions and Future Directions

T1D and AITD are complex diseases that are postulated to be caused by the combined effects of multiple susceptibility genes and environmental triggers. There are now solid epidemiological data showing that T1D and AITD frequently occur within the same families and in the same individual (APS3v). The association between T1D and AITD is most likely due to shared genetic predisposition (342). Indeed several loci and genes have been shown to contribute to the joint susceptibility to T1D and AITD (Table 5). Not unexpectedly, the joint susceptibility genes for T1D and AITD identified so far are all involved in the immune response. It is unlikely that shared autoantigenic epitopes contribute to the joint susceptibility to T1D and AITD, although such target organ “molecular mimicry” is still possible.

Subset analysis has been shown to be a powerful method to dissect the roles of “weak” susceptibility genes in complex disease (286). In many cases weak susceptibility genes predisposing to a broad genotype (*e.g.*, T1D or AITD) may have a strong effect in a subset, as has been clearly shown in the case of CTLA-4 and T1D (91, 92). However, it is possible that even within the subset of patients with T1D and AITD (*i.e.*, APS3v), there exist further subsets (*e.g.*, young age of onset, high level antibodies, and females) that will show specific genetic effects. With the completion of landmark projects in human genetics, including the human genome project, the HapMap project, and the mapping of most of the SNPs in humans, additional susceptibility genes for T1D and AITD will likely be identified in the near future. These will need to be tested in different subsets of patients such as T1D and AITD to clarify their role in the etiology of disease.

The next step in understanding the role of susceptibility genes in the etiology of T1D and AITD is to identify the causative variants and their effect on gene function and disease. This will enable us to dissect the gene-gene and genetic-epigenetic interactions leading to increased risk for disease. Some functional studies have been performed for HLA (293), CTLA-4 (161, 197, 330, 331), and PTPN22 (341). These functional studies suggest that abnormalities in antigen presentation and T cell activation might play a significant role in the shared genetic etiology of T1D and AITD.

In summary, significant progress has been made toward understanding the joint susceptibility genes for T1D and AITD. Dissecting the common genetic etiology of T1D and AITD will lead to a better understanding of the common mechanisms leading to autoimmunity in general and, hopefully, will lead to novel therapy and prevention modalities.

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