REVIEW

CTLA-4 and its role in autoimmune thyroid disease

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Abstract

Autoimmune thyroid disease (AITD) occurs in two common forms: Graves' disease and Hashimoto thyroiditis. On the basis of functional and experimental data, it has been suggested that the gene encoding cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) is a candidate gene for conferring susceptibility to thyroid autoimmunity. In this review, we critically evaluate the evidence for pathogenetic involvement of CTLA-4 in the various forms of AITD and focus on the possible role of genetic variation of the *CTLA4* locus. Population genetics data strongly suggest a role for the *CTLA4* region in susceptibility to AITD. However, further functional studies are required to understand the significance of *CTLA4* polymorphisms in the pathogenic mechanism of AITD.

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Introduction

Autoimmune thyroid disease (AITD) is an organ-specific disorder that affects the thyroid gland. It numbers among the most common human autoimmune diseases, affecting up to 5% of the general population (Wang & Crapo 1997). To detect the clinical symptoms of AITD, thyroid volume is usually determined with ultrasonography, and serum concentrations of three hormones [thyrotrophin (TSH), thyroxine (T_4) and triiodothyronine (T_3)] and autoantibodies against thyroid peroxidase (TPOab) and thyroglobulin (TGab) are measured.

Autoimmunity against the thyroid gland results in two opposite pathogenic paths: hyperthyroidism in Graves' disease and thyroid destruction in Hashimoto thyroiditis. Increased circulating activated T cells and thyroid-specific autoantibodies occur in Graves' disease. Antibodies directed against the TSH receptor stimulate thyroid function and lead to glandular overactivity (Kopp 2001). Depending on its severity, Graves' disease is

classified as subclinical or overt hyperthyroidism. Subclinical hyperthyroidism is characterised by increased serum TSH concentrations (more than 5 mIU/l) and normal T_3 (1.0–3.0 nmol/l) and T_4 (10–25 pmol/l) estimates. The overt disease is determined by increased TSH and thyroid hormone values (Faber *et al.* 2001, Fatourechi 2001).

Hashimoto thyroiditis is characterised by the loss of thyroid cells and gradual destruction of the gland, leading to thyroid hormone deficiency (Eguchi 2001). Subclinical Hashimoto thyroiditis is determined by a reduced serum TSH concentration (less than 0.3 mIU/ml) but with concentrations of free T_3 and T_4 hormones in the normal range. Under clinically presenting (overt) hypothyroidism, both TSH and thyroid hormone concentrations are decreased (Hueston 2001).

Postpartum thyroiditis is an autoimmune thyroid disorder affecting 5–9% of women during the years after delivery. The disease shares immunological and clinical features with autoimmune hypothyroidism and Graves' disease, and seems to

be caused by a combination of genetic and environmental factors (Lazarus *et al.* 2002). The immunological features of this disorder include the presence of TPOab (and, less commonly, TGab), abnormalities in the circulating T cell population and a goitre with lymphocytic infiltration, which are similar to the features of other forms of AITD (Stagnaro-Green 2000).

Extrathyroidal manifestations of AITD can affect the eyes and skin. Thyroid-associated ophthalmopathy (TAO) is clinically relevant in approximately 50% of patients with Graves' disease as Graves ophthalmopathy, but it may be found in patients with no past or present history of hyperthyroidism, or even in patients who are hypothyroid because of Hashimoto thyroiditis (Bartalena *et al.* 2000). Thyroid-associated ophthalmopathy is an inflammatory disease of the orbital tissues. The effects of inflammation, mediated through the release of cytokines, include proliferation of fibroblasts and adipocytes in the orbital soft tissues. The recruitment of T cells to the orbits of affected patients may result from the expression of the target of the aberrant immune response in Graves' disease – the TSH receptor – in the orbits of patients with these ophthalmic complications (Wiersinga & Bartalena 2002).

Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) has been shown to provide a negative signal to the T cell, thus limiting immune responses (Chambers & Allison 1997). CTLA-4 controls the adaptation of T cells to a state of proliferative unresponsiveness and tolerance – a fate that is alternative to inducing productive immunity (Bugeon & Dallman 2000). In addition, CTLA-4 signalling mediates antigen-specific apoptosis of T cells and suppresses autoreactive proliferation of T lymphocytes (Gribben *et al.* 1995). Therefore, this immunomodulatory protein is significant in regulating and maintaining self-tolerance. Breakdown of the tolerance may result in the induction of autoimmunity. T-lymphocyte-mediated destruction of host cells is one of the common pathogenic autoimmune mechanisms that include thyroid autoimmunity (Stassi & De Maria 2002). CTLA-4 affects downregulation of T cell function and therefore may have a crucial role in T-cell-mediated autoimmunity and hence in susceptibility to AITD (Karandikar *et al.* 1996).

In this review, we will discuss the role of CTLA-4 in thyroid autoimmunity. We will focus on the

genetics of CTLA-4 and the possible role of genetic polymorphisms of the *CTLA4* locus in predisposition to AITD.

Structure of the *CTLA4* gene and function of its protein product

The human *CTLA4* gene starts from 202 949·6 kb from the p-terminus of chromosome 2 and spans about 6·2 kb on chromosome region 2q33 (Dariavach *et al.* 1988). It exists as a single copy per haploid genome and consists of four exons. The first exon encodes the leader sequence of 37 amino acids, the second an immunoglobulin (Ig) V-like domain of 116 amino acids, the third a hydrophobic transmembrane region of 37 amino acids and the fourth a 34 amino acid cytoplasmic domain (Dariavach *et al.* 1988). Introns 1, 2 and 3 span 2·5, 0·5 and 1·1 kb respectively (Harper *et al.* 1991). The 5' region of the gene contains a Kozak consensus sequence with the ATG initiation codon, an in-frame stop codon 26 bp upstream of this ATG and a TATA box 75 bp upstream of the stop codon (Harper *et al.* 1991). The 3' untranslated region (UTR) is about 1·1 kb and comprises a stretch of almost 30 AT repeats (Harper *et al.* 1991). Two main human CTLA-4 transcripts of about 1·8 and 0·8 kb have been detected, the smaller of which may derive from the use of an alternate degenerated polyadenylation signal sequence (Harper *et al.* 1991).

The mature protein consists of 149 amino acids and is expressed exclusively in activated CD4(+) and CD8(+) T lymphocytes (Lindsten *et al.* 1993). However, an alternative splicing of the CTLA-4 mRNA has been described (Magistrelli *et al.* 1999). The alternative transcript has a deletion from nucleotide 456 (starting from the ATG codon) to nucleotide 563, resulting in the loss of exon 3 encoding 37 amino acids of the transmembrane domain and a frame-shift generating 22 extra amino acids before a translational termination at position 523 (Magistrelli *et al.* 1999). This spliced transcript produces a 23 kDa soluble form of CTLA-4, as opposed to a 45 kDa full-length transmembrane variant of the molecule (Oaks *et al.* 2000). Membrane CTLA-4 is expressed as a homodimer because of a disulphide bridge between cysteine residues at position 120. The soluble form lacks this cysteine residue, existing as a monomer

(Magistrelli *et al.* 1999). However, this CTLA-4 variant maintains the MUPPPY motif (critical for B7 molecule binding) and can therefore participate in the B7/CTLA-4/CD28 signalling pathway of T cell regulation. The soluble CTLA-4 is preferentially expressed in non-activated T cells (Oaks & Hallett 2000). In rat tissues, both forms of CTLA-4 have been detected in lymph node, spleen and peripheral blood, only full-length CTLA-4 in adult thymus, only short CTLA-4 in bone marrow cells and neither form in non-lymphoid tissues (Oaks *et al.* 2000). The short splice variant of CTLA-4 was observed significantly more often in patients with AITD in comparison with non-affected individuals (Oaks & Hallett 2000). This finding will be discussed below.

The human *CTLA4* and *CD28* genes are closely linked. They are separated by only 130 kb (Buonavista *et al.* 1992). CTLA-4 and CD28 are very similar at the message and gene structure levels. These homologies and gene proximity strongly suggest that CTLA-4 and CD28 are the direct products of a duplication event (Balzano *et al.* 1992). However, 3' and 5' flanking sequences of these genes have no homology, suggesting therefore that different regulatory mechanisms for the two genes might be functioning.

CTLA-4 and CD28 act as members of the same pathway of T cell regulation. The CTLA-4 molecule binds the same ligands as CD28 [B7-1 (CD80) and B7-2 (CD86)], but with at least a 10-fold greater affinity (Greene *et al.* 1996). CD28 is expressed both in resting and activated T cells (Leung & Linsley 1994). Binding of the costimulatory B7 molecule to CD28 activates T cells and the immune response by inducing the expression of several cytokines, cytokine receptors and regulatory genes (Leung & Linsley 1994). In contrast to CD28, CTLA-4 acts as negative regulator of T cell function and autoreactivity.

CTLA-4 can downregulate T cell responses by two separate mechanisms. One mechanism is CTLA-4-mediated negative signalling in response to T cell receptor activation (Lee *et al.* 1998). This mechanism requires the cytoplasmic tail of CTLA-4 and can occur in early stages of an immune response when expression of CTLA-4 and B7 is limited (Carreno *et al.* 2000). The other mechanism operates through cell-surface competition between CTLA-4 and CD28 for B7 binding. This mechanism depends on the levels of surface

expression of CTLA-4 (Carreno *et al.* 2000). Both mechanisms can occur in late stages of the immune response when there is increased expression of B7 and CTLA-4. Binding of B7 to CTLA-4 leads to termination of the immune response via limitation of CD28-mediated signalling, T cell anergy and T cell apoptosis (Perez *et al.* 1997, Oosterwegel *et al.* 1999). CTLA-4-deficient mice exhibit massive lymphoproliferative autoimmune disease (Tivol *et al.* 1995, Waterhouse *et al.* 1995). These observations suggest that the CTLA-4/B7 interaction has a key role in maintaining the peripheral immune tolerance status, and hence in autoimmunity (Tivol *et al.* 1996).

Polymorphic markers within the *CTLA4* gene

The *CTLA4* locus has three polymorphic markers, all of which have been examined in AITD. Firstly, there is a cytosine to thymine substitution at position -318 [C(-318)T] of the promoter region (Deichmann *et al.* 1996). This polymorphism can easily be detected by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach using treatment with MseI restriction endonuclease (Deichmann *et al.* 1996). The C(-318)T variation has a heterozygosity of approximately 0.25 and a polymorphism information content of 0.15.

The second nucleotide variation, an adenine to guanine transition, occurs at position 49 (A49 G) of exon 1 and leads to alanine to threonine amino acid substitution of codon 17 of the leader peptide (A17T) (Nistico *et al.* 1996). The AT17 dimorphism can be tested by a PCR-RFLP-based assay using BstEII restriction endonuclease (Marron *et al.* 1997). Heterozygosity is approximately 0.45 and it has a polymorphism information content of 0.25.

The third polymorphic marker is located in the 3' UTR of exon 4 (Harper *et al.* 1991). This (AT)_n dinucleotide repeat polymorphism starts at nucleotide 642 of exon 4 of the human *CTLA4* gene (Dariavach *et al.* 1988). The marker is highly polymorphic: at least 23 different alleles containing from seven to 30 AT repeats can be detected by PCR (Polymeropoulos *et al.* 1991). The alleles are numbered in several ways: some authors have used mobility units based on automated fragment length analysis; others have estimated the length on the basis of the size of PCR products - 106 bp, 108 bp, etc. Finally, some authors have used the number of

A repeats. The (AT)_n repeat dinucleotide marker is highly informative, with a heterozygosity of 0.93 and a polymorphism information content of 0.91.

Strong linkage disequilibrium has been demonstrated between the promoter and exon 1 polymorphisms. In Germans, the T(−318) allele was strongly linked to the A49 allele (Donner *et al.* 1998), whereas a linkage of the allele C(−318) to the G49 allele was found in Koreans (Park *et al.* 2000). Evidence has been obtained for linkage between the G49 allele and the 106 bp allele of the (AT)_n repeat polymorphism in some populations (Nistico *et al.* 1996, Larsen *et al.* 1999, Kouki *et al.* 2002).

The functional significance of the *CTLA4* polymorphisms remains unclear. The (AT)_n repeat may affect RNA stability; this ability has been demonstrated for AT-rich sequences in 3' UTRs of some genes (Shaw & Kamen 1986). The dinucleotide polymorphism occurs in the vicinity of an intracellular localisation motif that is situated in the middle of exon 4 (Leung *et al.* 1995). Contained within this motif are a binding site and a phosphorylation signal for the SH2 domain containing tyrosine phosphatases (Leung *et al.* 1995). The T cell receptor and CTLA-4 both interact with this signal transduction pathway, but no studies relating these data to the *CTLA4* polymorphisms are available at present.

In Swedish patients, a relationship between both the promoter and the exon 1 polymorphisms and the level of expression of CTLA-4 has been found (Ligers *et al.* 2001). A correlation between the codon 17 dimorphism and CTLA-4 mRNA levels has also been shown in peripheral lymphocytes from Canadian individuals (Barnes *et al.* 1997). However, no correlation between the A49 G dimorphism or the dinucleotide microsatellite and gene expression was observed in an other study (Cavallo *et al.* 1997). Meanwhile, recent studies have shown a relationship between the A49 G variation and the strength of downregulation of T cell activation (Kouki *et al.* 2000, Maurer *et al.* 2002). The exon 1 polymorphism was found to affect the inhibitory function of CTLA-4 and the G49 allele was associated with reduced control of T cell proliferation (Kouki *et al.* 2000). The intracellular distribution of CTLA-4 demonstrated qualitatively different staining patterns between patients with the G/G genotype and individuals homozygous for A at position 49 (Maurer *et al.*

2002). These findings are extremely important to the understanding and assessment of a role for different molecular variants of CTLA-4 in the pathogenesis of thyroid and other autoimmune diseases.

The *CTLA4* gene and susceptibility of disease

The *CTLA4* gene and susceptibility to Graves' disease

The first evidence for association between the (CA)_n dinucleotide repeat polymorphism at the 3' UTR of the *CTLA4* gene and Graves' disease in white North Americans was reported in 1995 (Yanagawa *et al.* 1995). Association of the three polymorphic markers of the *CTLA4* gene with the disease was demonstrated in most population-based investigations (Table 1). The G49 allele of the codon 17 dimorphism has been shown to be a common genetic risk marker of Graves' disease in different populations. For the polymorphic (CA)_n repeat, an association of the 106 bp allele with a greater risk for the disease was detected in several studies (Kotsa *et al.* 1997, Hadj Kacem *et al.* 2001, Kouki *et al.* 2002). The *CTLA4* locus is the only non-human leucocyte antigen (*HLA*) locus for which association with Graves' disease has been demonstrated repeatedly.

In 1999, a linkage to Graves' disease was also found using a set of 66 multiplex British families (Vaidya *et al.* 1999b). The *CTLA4* locus located within a 10 cM region of chromosome 2q31-q33 between markers *D2S389* and *D2S116* showed a maximum strength of linkage with the disorder (Vaidya *et al.* 1999b). The linkage of *CTLA4* to Graves' disease was confirmed using an enlarged set of 179 UK families (Heward *et al.* 1999). However, family studies showed no linkage between the *CTLA4* locus and Graves' disease in other populations (Maalej *et al.* 2001, Sakai *et al.* 2001, Nithiyananthan *et al.* 2002, Tomoyose *et al.* 2002, Villanueva *et al.* 2002). It thus remains questionable whether the *CTLA4* gene is linked to the disease.

Without doubt *CTLA4* is strongly associated with Graves' disease. In white Britons, the *CTLA4* locus and the *HLA* locus together confer up to 50% of the inherited susceptibility to the disease (Vaidya

Table 1 Inventory of genetic studies of the CTLA4 polymorphisms in Graves' disease

Patients (No.)	Controls (No.)	Families (No.)	Population	Polymorphism			3' (AT) _n	P	Reference
				C(-318)T	P	A49G			
133	85		US white				Genotype	0.012	Yanagawa <i>et al.</i> (1995)
94	77		Hong Kong				Genotype		Nistico <i>et al.</i> (1996)
112	91		UK				106 bp	0.006	Kotsa <i>et al.</i> (1997)
153	200		Japanese				Allele	<0.01	Yanagawa <i>et al.</i> (1997)
28	94		Chinese				Genotype	NS	Marron <i>et al.</i> (1997)
305	325		German				Allele	NS	Donner <i>et al.</i> (1997b)
125	142		German	C/C	0.006	Genotype			Braun <i>et al.</i> (1998)
				C/T	0.008				
		109 simplex	German	C-allele	NS	G-allele	84 bp	<0.05	Donner <i>et al.</i> (1998)
				T-allele	NS	A-allele	102 bp	NS	
191	355		UK	C-allele	NS		118 bp	<0.04	Heward <i>et al.</i> (1998)
				T-allele	NS				
		78 simplex	UK	T-allele	NS				
98	82		Hong Kong	C-allele	NS				
				T-allele	NS				
		13 simplex	Hong Kong	T-allele	NS				
112	425		Japanese						Awata <i>et al.</i> (1998)
73	100		French			Genotype			Djilali-Saiah <i>et al.</i> (1998)
379	363		UK			G-allele			Heward <i>et al.</i> (1999)
						A-allele			
		179 multiplex				Allele			
		66 multiplex				A/A			
			UK			G/G			
						G-allele			
			UK			Linkage			
						(G-allele)			
			Russian			Linkage	Linkage (112 mu)	0.02	Vaidya <i>et al.</i> (1999b)
						(G-allele)			Chistyakov <i>et al.</i> (2000)
78	93		Japanese			Allele			Akamizu <i>et al.</i> (2000)
156	118		US			Genotype	102 bp	<0.01	Kouki <i>et al.</i> (2000)
45	43						104 bp	<0.01	Park <i>et al.</i> (2000)
97	199		Korean			Allele			Hadi Kacem <i>et al.</i> (2001)
144	205		Tunisian	C-allele	<0.05	G-allele	Genotype	<0.0084	Kouki <i>et al.</i> (2002)
				C/C	<0.05	G/G	106 bp	<0.001	Yung <i>et al.</i> (2002)
120	80		US	Allele	NS	Allele	106 bp	<0.01	
123	158		Chinese	Genotype	NS	A/A			
				Allele		G-allele			
						Allele			
						G-allele			

mu, mobility units; NS, not significant.

et al. 1999b). The region of linkage to Graves' disease on chromosome 2q31-q33 is close to a locus of susceptibility to insulin-dependent diabetes mellitus (IDDM), *IDDM12* (Vaidya *et al.* 1999b). *CTLA4* is the most likely known candidate gene for *IDDM12* on 2q33 (Nistico *et al.* 1996, Marron *et al.* 1997, She & Marron 1998). Besides the association of the *CTLA4* locus with IDDM and Graves' disease, relationships between this locus and other autoimmune diseases such as multiple sclerosis, Addison's disease, rheumatoid arthritis and systemic lupus erythematosus have been demonstrated (Kristiansen *et al.* 2000). Thus the *CTLA4* locus might be established as the first known major non-*HLA* locus of human autoimmunity.

The *CTLA4* gene and susceptibility to Hashimoto thyroiditis

A significant association between the *CTLA4* gene and Graves' disease has been found in several populations. In contrast, there are few studies that have examined whether this gene is associated with Hashimoto thyroiditis (Table 2). Kotsa *et al.* (1997) reported that the dinucleotide microsatellite of the *CTLA4* gene is associated with autoimmune hypothyroidism in the British population. Other investigators have obtained evidence of association with the disease for the (AT)_n repeat polymorphism (Sale *et al.* 1997) and the codon 17 dimorphic site (Donner *et al.* 1997a, Awata *et al.* 1998, Barbesino *et al.* 1998, Tomer *et al.* 1999), but never for the C(−318)T substitution in the promoter region of the *CTLA4* gene (Braun *et al.* 1998, Heward *et al.* 1998, Tomer *et al.* 1999, Park *et al.* 2000, Tomoyose *et al.* 2002). In the case of Graves' disease, an association has been demonstrated for all three polymorphisms. However, too few data are available to permit the conclusion that the promoter polymorphism is not associated with Hashimoto thyroiditis. Further population studies are required to determine whether this single nucleotide polymorphism is related to the disorder.

Analysis of 48 multiplex families, of which 46 were white, 20 had at least two members with Hashimoto thyroiditis and 17 had one sibling affected by Hashimoto thyroiditis, showed no linkage between the *CTLA4* (AT)_n repeat polymorphism and autoimmune hypothyroidism (Nithiyananthan *et al.* 2002). A whole-genome scan

using a subset of 42 families (22 comprising only Hashimoto thyroiditis-affected members, and 20 with one child affected by Hashimoto thyroiditis) from 56 white multiplex AITD families mapped three regions of linkage with autoimmune hypothyroidism on chromosome 6p (*AITD-1* susceptibility locus), 12q22 [Hashimoto thyroiditis (*HT*)-1 locus] and 13q22 (*HT*-2 locus), and showed no linkage to chromosome 2q33, where the *CTLA4* gene is located (Tomer *et al.* 1999). A recent genome-wide analysis using data from 123 Japanese sib-pairs affected with AITD found a new Hashimoto thyroiditis susceptibility locus at chromosome 8q23-q24, but not at 2q33 (Sakai *et al.* 2001). These data thus suggest that the *CTLA4* gene is probably not linked to Hashimoto thyroiditis. However, there seems to be some evidence supporting an association between the *CTLA4* locus and Hashimoto thyroiditis.

The *CTLA4* gene and susceptibility to autoimmune thyroid disease

Case-control studies on the association between the *CTLA4* gene and AITD have been performed in Japan only, and their results are conflicting (Sale *et al.* 1997, Akamizu *et al.* 2000). No evidence was found for linkage between the *CTLA4* gene and AITD (Nithiyananthan *et al.* 2002) in studies that included two genome-wide scans (Tomer *et al.* 1999, Sakai *et al.* 2001) and analyses of large Tunisian (Maalej *et al.* 2001) and Chinese pedigrees (Villanueva *et al.* 2002).

Meanwhile, a number of investigations support a relation between the *CTLA4* gene and features of autoimmune thyroid disease such as production of thyroid autoantibody (Tab). The G49 allele was found to be associated with greater concentrations of Tab and to be linked to Tab production, including production of TGAb and TPOAb (Tomer *et al.* 2001, Zaletel *et al.* 2002). A susceptibility locus for Tab production was mapped on chromosome 2q33 around marker *D2S155*, which is linked to the region containing the *CTLA4* and *CD28* genes (Tomer *et al.* 2001). Further analysis showed that a major gene for production of Tab on chromosome 2q33 is most probably the *CTLA4* gene and not the *CD28* gene (Tomer *et al.* 2001). However, there was still no evidence that *CTLA4* contributes specifically to Graves' or Hashimoto diseases.

Table 2 Inventory of genetic studies of the CTLA4 polymorphisms in Hashimoto thyroiditis

Patients (No.)	Controls (No.)	Families (No.)	Population	Polymorphism			Reference
				C(-318)T	P	A49G	
44	91		UK				Kotisa <i>et al.</i> (1997)
73	466		German + Canadian			Genotype	Donner <i>et al.</i> (1997a)
45	113		Japanese			Allele	Sale <i>et al.</i> (1997)
92	355		UK	C-allele	NS		Heward <i>et al.</i> (1998)
				T-allele	NS		
88	425	6 simplex	UK	T-allele	NS		
			Japanese			Genotype	Awata <i>et al.</i> (1998)
						G-allele	
64	142		German	C/C	NS	A-allele	Braun <i>et al.</i> (1998)
				C/T	NS		
158	384		UK			G-allele	Barbesino <i>et al.</i> (1998)
						G/G + A/G	
143	199		Japanese	Allele	NS	G-allele	Tomer <i>et al.</i> (1999)
110	199		Korean	Allele	NS	Allele	Park <i>et al.</i> (2000)
183	118		Japanese				Akamizu <i>et al.</i> (2000)
126	301		Italian			Genotype	Petrone <i>et al.</i> (2001)
99	91		Japanese	Allele	NS	G-allele	Tomoyose <i>et al.</i> (2002)
				Genotype	NS	G/G	
				CG allele	0-0216		
				haplotype			
				CG/CG	0-0106		
				haplotype			

NS, not significant.

The *CTLA4* gene and susceptibility to other forms of autoimmune thyroid disease

The codon 17 dimorphism of the *CTLA4* gene has been tested for association with thyroid-associated ophthalmopathy. Buzzetti *et al.* (1999) and Vaidya *et al.* (1999b) found an association of the G49 allele with increased risk of Graves' disease in white patients in the UK and Italy respectively, whereas the findings of other case-control studies were negative (Table 3). For polymorphisms of other immune response genes, conflicting results have been reported (Farid & Balasz 1998, Buzzetti *et al.* 1999, Hunt *et al.* 2000). The segregation ratio for ophthalmopathy in families with Graves' disease was shown to be zero, suggesting that environmental factors rather than genetic factors predispose to the development of this ophthalmopathy (Villanueva *et al.* 2000, Bednarczuk *et al.* 2003). Therefore, the *CTLA4* polymorphism is probably not associated with thyroid-associated ophthalmopathy. However, Vaidya *et al.* (1999b) showed a maximum association strength of the G49 allele (odds ratio 3.06) with severe forms of the disease, suggesting that genetic factors could contribute more significantly to the severity of thyroid-associated ophthalmopathy.

One investigation concerned the association of the *CTLA4* gene with postpartum thyroiditis only. No significant association between the 106 bp allele of the polymorphic (AT)_n repeat (which is in linkage disequilibrium with the G49 allele) and the disease was observed in the Welsh population (Waterman *et al.* 1998). More studies are therefore required for estimating the genetic role of the *CTLA4* locus in the aetiology of postpartum thyroiditis.

Possible reasons for the inconsistencies between disease states and *CTLA4* genotype

Among the different forms of AITD, the polymorphisms of the *CTLA4* gene showed strong association with Graves' disease, demonstrated conflicting findings in Hashimoto thyroiditis patients and rather a lack of association with thyroid-associated ophthalmopathy. Difference in definition of the disease is one factor that can cause the discrepancies between case-control studies. Variability in the normal range of TSH and thyroid hormone concentrations is not very marked between different investigations, but significant

dispersal occurs in AITD-defined estimates of serum anti-thyroid autoantibodies (50–200 U/ml for TGab, 10–60 U/ml for TPOab and 9–15 U/l for antibodies against the TSH receptor).

Another possible reason for inconsistencies between disease states and *CTLA4* genotype may lie in significant variations in the ratio of women to men in the case groups tested (from 2.5 to 14.8) compared with the controls (from 0.9 to 3.5).

The codon 17 amino acid substitution has been shown to be functionally relevant (Maurer *et al.* 2002) and has been tested significantly more often than the two other *CTLA4* polymorphisms. It seems that the A49 G dimorphism allele frequencies do not change significantly in relation to age and sex, but can vary markedly in relation to ethnic background: the A49 allele dominates in whites, whereas the G49 allele is most common in Asian populations (Yung *et al.* 2002). However, patients in almost all case-control studies were ethnically matched and an association with the disease was observed in both whites and Asians. Ethnic and regional heterogeneity may therefore contribute weakly to explaining the differences between association studies.

Using equations reported by Ohashi *et al.* (2001) and Tsuchiya *et al.* (2002), it is possible to estimate the number of cases needed to obtain a statistical power of $1-\beta$ with a significance level of α . We can take two extreme examples, based on the size of the sample tested. Heward *et al.* (1999) showed a significant association between the A49 G dimorphic site of the *CTLA4* gene and Graves' disease ($P < 0.00003$), studying 379 affected patients and 363 controls. For this study, at least 151 cases are required to obtain 80% power with a significance level of 0.05 in a two-sided test; Heward's group studied a case sample 2.5 times larger than this. In contrast, Kouki *et al.* (2000) analysed only 45 patients with Graves' disease and 43 healthy individuals; nevertheless they also found an association. In their study, at least 46 affected individuals should have been examined to achieve the significance level mentioned above. Thus the association found by Kouki *et al.* (2000) is questionable rather than true. Other investigators have analysed samples of sizes intermediate between these extreme examples, which should be adequate to reveal significant associations.

However, finding an association does not always mean finding a susceptibility gene. Most of the

Table 3 Inventory of genetic studies of the CTLA4 polymorphisms in thyroid-associated ophthalmopathy

Patients (No.)	Controls (No.)	Families (No.)	Population	Polymorphism				Reference
				C(-318)T	P	A49G	P	
135	124		Germany			A/A	NS	Badenhoop <i>et al.</i> (1996)
94	94		UK			G/G + A/G	NS	Vaidya <i>et al.</i> (1999a)
38	54		Italian			G/G + A/G	0.012	Buzzetti <i>et al.</i> (1999)
85	52		US white			G/G + A/G	0.027	Villanueva <i>et al.</i> (2000)
						Allele	NS	
323	161		UK			Genotype	NS	Allahabadia <i>et al.</i> (2001)
						Allele	NS	
95	169		Polish			Genotype	NS	Bednarczyk <i>et al.</i> (2003)
						Allele	NS	
99	220		Japan			Genotype	NS	
						Allele	NS	
						Genotype	NS	

NS, not significant.

susceptibility genes with genotype relative risks greater than 2.0 and a frequency of the disease allele of more than 0.2 can be identified with a power of 0.8 using about 500 cases and 500 controls (Ohashi *et al.* 2001). If the sample size for each group is about 100 and the frequency ranges between 0.1 and 0.4, a genotype relative risk of more than 3.0 is required to detect the susceptibility gene with 80% statistical power. For the *G/G* homozygous genotype, a relative risk value ranging between 2.0 and 2.8 was obtained in most case-control studies using the A49 G polymorphism to detect association between the *CTLA4* gene and thyroid autoimmunity. Thus it remains unclear whether the *CTLA4* gene is a true AITD susceptibility gene or whether the polymorphisms within this gene are in strong linkage disequilibrium with a nearby gene that implicates susceptibility to AITD.

Functional significance of the *CTLA4* gene polymorphisms and their possible applications in the pathogenesis of thyroid autoimmunity

Role of CTLA-4 in the pathogenic mechanism of autoimmune thyroid disease

Among all three known polymorphic markers of the *CTLA4* gene, a functional significance is only more or less clear for the codon 17 polymorphism. Because of the relationship of the G49 allele to a greater risk for AITD, *G/G* homozygous patients might be expected to have a weak suppressing function of the Ala17 molecular variant of CTLA-4 in comparison with the *A/A* genotype. Disruption of the precise balance between CD28 and CTLA-4 interactions with B7 could lead to autoimmune disease by preventing apoptosis or downregulation of activated T cells (Tivol *et al.* 1996). Hence, aberrant T cell clones can survive in the thymus and produce autoantibodies against the TSH receptor and other thyroid autoantigens that could initiate autoimmune thyroid disease (Shimojo *et al.* 1996). This idea is supported by the data of Kouki *et al.* (2000), who found that the G49 allele is associated with reduced control of T lymphocyte proliferation.

Recent findings suggest that levels of surface expression of CTLA-4 and its intracellular distribution correlate with a genotype at position 49

(Maurer *et al.* 2002). Individuals carrying the *G/G* genotype had reduced upregulation of surface expression of CTLA-4, whereas activated T cells from patients homozygous for adenine revealed a more circular and homogeneous cytoplasmic pattern of staining for CTLA-4 localisation compared with that in *G/G* homozygotes (Maurer *et al.* 2002). A relationship between the genotype and level of expression of CTLA-4 was demonstrated in other recent investigations (Ligers *et al.* 2001). Specifically, individuals carrying thymine at position -318 of the *CTLA4* promoter and homozygous for adenine at position 49 in exon 1 showed significantly increased expression both of cell-surface CTLA-4 after cellular stimulation and of CTLA-4 mRNA in non-stimulated cells (Ligers *et al.* 2001).

The majority of CTLA-4 molecules occur within the cytoplasm and can be quickly mobilised from intracellular store compartments to the site of T cell receptor engagement on the cell surface (Linsley *et al.* 1996). Hence, low concentrations of intracellular CTLA-4 may correlate with decreased cell surface expression of CTLA-4 and therefore with reduced negative control of T cell proliferation. The *CTLA4* A49 G single nucleotide polymorphism results in the substitution of threonine with alanine at codon 17 of the leader sequence. This sequence serves as a signal peptide to direct the secreted protein to the endoplasmic reticulum. The codon 17 amino acid variation can affect a conformation of the leader peptide that leads to an 'altered address' of intracellular CTLA-4 trafficking. This might result in altered transition of CTLA-4 molecules between intracellular pools and the cell surface. So, carriers of the Ala17 molecular variant of CTLA-4 should exhibit less effective transition of intracellular CTLA-4 to the T cell surface (Simon & Blobel 1991). This might correlate with decreased negative regulation of T cell proliferation and therefore predispose to greater risk of development of AITD.

However, Xu *et al.* (2002) showed that there was no differential effect of the A49 and G49 alleles on the expression and function of recombinant human CTLA-4 *in vitro*. Hence, these data mean that the functional relevance of the codon 17 polymorphism remains questionable and suggest that cell membrane CTLA-4 may be not directly involved in the pathogenesis of autoimmune disease. Meanwhile, the A49 G substitution has been shown to be in

linkage disequilibrium with other polymorphic sites within the *CTLA4* gene that can be functionally significant (Donner *et al.* 1998, Park *et al.* 2000, Kouki *et al.* 2002).

Some interesting investigations were carried out to clarify the functional relevance of these polymorphic sites. It has been shown that the presence of thymine at position -318 of the *CTLA4* promoter and adenine at position 49 in exon 1 is significantly correlated with greater expression, both of cell-surface CTLA-4 after cellular stimulation and of CTLA-4 mRNA in non-stimulated T cells (Ligers *et al.* 2001). Enlarging the length of the 3' UTR (AT)_n microsatellite of the allele has been observed to correlate with T cell hyperactivity *in vitro* and can result in increased instability of CTLA-4 mRNA (Huang *et al.* 2000). Further functional genomics studies are required to derive an in-depth understanding of the significance both of the C(-318)T promoter and the 3' UTR dinucleotide polymorphism in the molecular mechanism of autoimmunity.

Another possibility may lie in the alternately spliced form of CTLA-4 (Magistrelli *et al.* 1999, Giorelli *et al.* 2001). In contrast to the full-length membrane CTLA-4, this molecular variant of CTLA-4 (soluble CTLA-4, or sCTLA-4) lacks the transmembrane domain and can be constitutively expressed by non-activated T cells (Oaks *et al.* 2000). The sCTLA-4 is found to be significantly more frequent in patients affected with autoimmune thyroid disease than in healthy individuals (Oaks & Hallett 2000). A role of this CTLA-4 form in AITD is unknown and is being predicted only. The soluble CTLA-4 has a B7 recognition site and therefore can successfully compete with the membrane CTLA-4 for B7 binding. Increased concentrations of sCTLA-4 could impede the full-length CTLA-4 from performing its protective role against autoimmune reactive T cell clones and from contributing in this way to the development of thyroid autoimmune pathology. In this regard, it is interesting that the sCTLA-4 does not appear to be expressed within the thymus, an organ in which negative selection of autoreactive T lymphocytes occurs (Oaks *et al.* 2000).

Finally, there are several possible mechanisms for CTLA-4-directed genetic effects in the modulation of the development of AITD. Two or more

polymorphisms of the *CTLA4* gene might be responsible for this modulation and these polymorphisms may cosegregate in a common risk haplotype (Tomer 2001).

How many genes in the *CTLA4* region can predispose to the autoimmune thyroid disease?

Population genetic data clearly suggest a role for the *CTLA4* region in susceptibility to AITD: we have strong evidence for the association between the *CTLA4* gene and thyroid autoimmunity. Nevertheless, it remains unclear whether the *CTLA4* locus is linked to the disease, because linkage between the *CTLA4* gene and Graves' disease has been demonstrated in the British population only (Heward *et al.* 1999, Vaidya *et al.* 1999b).

AITD seems to be a polygenic disease with no major susceptibility locus. The *CTLA4* gene has been shown to be a susceptibility factor that is separate from the *HLA* locus in Graves' disease and Hashimoto thyroiditis (Yanagawa *et al.* 1995, Donner *et al.* 1997a,b). The *CTLA4* region and the *HLA* locus have been established as the loci that predispose to AITD, and each of them has a minor but significant effect (Tomer & Davies 1997). Using non-parametric linkage analysis provides a genome-wide search for the susceptibility regions, but has low power of detection to identify loci with relatively small genetic effects, whereas case-control association studies have strong advantages for the detection of such loci (Tsuchiya *et al.* 2002). All previous whole-genome scans searching for the loci of AITD susceptibility dealt with family sets of relatively small numbers (Tomer *et al.* 1999, Sakai *et al.* 2001). It would be necessary to enlarge the data set significantly in order to have sufficient power to reveal linkage between the disease and genomic regions with minor genetic effects.

The G49 *CTLA4* allele has been found to be associated with greater levels of thyroid antibodies and to be linked to Tab production, including production of TGab and TPOab (Tomer *et al.* 2001, Zaletel *et al.* 2002). Hence, *CTLA4* might be the susceptibility gene for thyroid autoantibody production, and the G49 allele may predispose individuals to develop Tab. Alternatively, the *CTLA4* codon 17 polymorphism might be in strong linkage disequilibrium with another causative gene

that implicates susceptibility to Tab production. It does not exclude the possibility that there are two closely linked genes (one of them likely to be *CTLA4*) accounting for the susceptibility to produce autoantibodies against the thyroid.

The *CTLA4* gene is located in a chromosome region 2q33 that contains the genes encoding two other T lymphocyte costimulatory receptors, CD28 and ICOS (Ling *et al.* 2001). The *CD28* gene has been excluded as the putative susceptibility gene in this region (Marron *et al.* 2000, Tomer *et al.* 2001). The *ICOS* gene might be a likely candidate for Tab production; it encodes an inducible T cell costimulator and is separated from the *CTLA4* gene by 69 kb (Coyle *et al.* 2000, Ling *et al.* 2001).

The *ICOS* gene is a member of the family of CD28 and CTLA-4 cell-surface receptors and shares 24% and 17% identity with the CD28 and CTLA-4 amino acid sequences respectively (Hutloff *et al.* 1999). ICOS matches CD28 in potency and enhances all basic T cell responses to a foreign antigen – namely proliferation, secretion of lymphokines, upregulation of molecules that mediate cell–cell interaction, and effective help for antibody secretion by B cells (Dong *et al.* 2001). The *ICOS* gene is closely linked to the *CTLA4* gene and therefore might interfere with the contribution of *CTLA4* to susceptibility to thyroid antibody production.

It has recently been found that CTLA-4 is able to inhibit ICOS-mediated costimulation and prevent ICOS-costimulated T cells from producing interleukins-4, -10 and -13 (Riley *et al.* 2001). This suggests an alternative mechanism of CTLA-4 function, disruption of which could also predispose to autoimmunity. Therefore, the *ICOS* gene might be considered to be another candidate for susceptibility gene of thyroid autoimmunity. Some polymorphisms have been detected in non-coding regions of the *ICOS* gene (Haimila *et al.* 2002, Ling *et al.* 2001). One of these polymorphisms, the dinucleotide microsatellite located in intron 4, was tested but showed no association with autoimmune diabetes in a Japanese population (Ihara *et al.* 2001). More genetic and functional studies of *ICOS* are needed to identify clearly its role in autoimmunity.

Other possible candidates may be found among members of the *CFLAR-CASP10-CASP8* gene cluster that is positioned about 2.7 Mb distal to the *CD28-CTLA4-ICOS* gene cluster (Grenet *et al.*

1999). This cluster encodes caspase 8 and FADD-like apoptosis regulator (CFLAR) and caspases 8 and 10. Both caspases have shown to be involved in CD95-mediated cell apoptosis (Sprick *et al.* 2002). Interactions between CD95 and its ligand mediate thyrocyte destruction in Hashimoto thyroiditis (Stassi *et al.* 2000, Eguchi 2001). Caspases and death receptors/ligands would appear to play a part in CD95-induced apoptosis of Hashimoto thyroiditis thyrocytes (Hammond *et al.* 2001, Stassi & De Maria 2002). In addition, mutations in the *CASP10* gene lead to breakdown of lymphocyte homeostasis and normal immunological tolerance, and lead to the development of autoimmune lymphoproliferative syndrome (Jackson & Puck 1999). These findings favour the caspase-encoding genes as candidates for thyroid autoimmunity susceptibility gene(s).

Conclusions

CTLA4 as a thyroid autoimmunity susceptibility gene cannot explain the full genetic susceptibility to AITD. This gene has a significant but relatively small effect, and does not seem to determine the phenotypic expression of thyroid autoimmunity such as Graves' disease, Hashimoto thyroiditis and production of Tabs (Tomer 2001).

AITD can often occur in conjunction with other autoimmune disorders (IDDM, vitiligo, multiple sclerosis, Addison's disease, rheumatoid arthritis, systemic lupus erythematosus and others). Familial association studies have also reported an increased risk of several systemic autoimmune diseases among relatives of patients with a systemic autoimmune disease. This association may reflect a common aetiological pathway with shared genetic or environmental influences among these diseases (Cooper *et al.* 1999, Devendra & Eisenbarth 2003). There is also evidence that many autoimmune diseases share a common set of susceptibility genes.

In the case of *CTLA4* gene polymorphisms, an association has been observed with several autoimmune disorders (Kristiansen *et al.* 2000). CTLA-4 controls common immunity traits such as the amplitude of the immune response and peripheral tolerance, disruption of which can cause autoimmune disease. Hence, the *CTLA4* gene contributes to autoimmunity in general, but cannot account for determining the organ-specificity of

autoimmunity, and its role in the predisposition to AITD is rather non-specific (Rapoport & McLachlan 2001, Einarsdottir *et al.* 2003, Weetman 2003).

Genetic susceptibility factors in familial forms of complex diseases such as AITD and IDDM may be constituted by the combined effect of minor risk factors, accumulating in a greater-risk haplotype. Therefore, susceptibility loci with low genetic effects (such as *CTLA4*) could be successfully identified through genetic analysis of large pedigrees (Einarsdottir *et al.* 2003).

Further genetic and functional experiments should be undertaken to estimate the significance of the *CTLA4* gene in thyroid autoimmunity. In this respect, the use of animal models would help to increase the power of linkage analysis to locate susceptibility genes with minor effects, because of the complete nature of the information to be derived from all matings (all parents are heterozygous in an intercross) and the reduction of genetic complexity in inbred strains (Lam-Tse *et al.* 2002). Secondly, animals affected by autoimmune diseases provide a unique opportunity for a precise understanding of the early stages of the endocrine autoimmune process and the identification of disease-associated pathways, which are complicated to define in man. Thirdly, animal models are extremely helpful for testing and studying the treatment effects of CTLA4-Ig, a new immunosuppressive therapeutic agent that is more and more widely used for inhibition of T-cell-dependent antibody responses to slow progression of autoimmune disease, to induce long-term donor-specific tolerance and to prolong the survival of transplanted organ in recipient patients (Najafian & Sayegh 2000).

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