European Journal of Endocrinology (2004) **150** 619–626 ISSN 0804-4643

REVIEW

The emerging role of the *CTLA-4* gene in autoimmune endocrinopathies

Bijay Vaidya and Simon Pearce[1]

Department of Diabetes, Endocrinology and Vascular Medicine, Peninsula Medical School, Royal Devon and Exeter Hospital, Exeter EX2 5DW, UK and [1]School of Clinical Medical Sciences and Institute of Human Genetics, Newcastle University, Newcastle upon Tyne, UK

(Correspondence should be addressed to B Vaidya; Email: bvaidya@hgmp.mrc.ac.uk)

Abstract

It is thought that the majority of autoimmune endocrinopathies, including Graves' disease, autoimmune hypothyroidism, type 1 diabetes mellitus and autoimmune Addison's disease (sporadic and as well as autoimmune polyendocrinopathy syndrome type 2) are inherited as complex genetic traits. Multiple genetic and environmental factors interact with each other to confer susceptibility to these disorders. In recent years there have been considerable efforts towards defining susceptibility genes for complex traits. These investigations have shown, with increasing evidence, that the cytotoxic T lymphocyte antigen-4 (*CTLA-4*) gene is an important susceptibility locus for autoimmune endocrinopathies and other autoimmune disorders. Here we review the genetic and functional analyses of the *CTLA-4* locus in autoimmune endocrinopathies, and discuss the recent efforts in fine-mapping this locus.

European Journal of Endocrinology **150** 619–626

Introduction

Autoimmune endocrinopathies, including Graves' disease (GD), autoimmune hypothyroidism (AH), type 1 diabetes mellitus (T1DM) and autoimmune Addison's disease (AAD) together affect up to 3% of the general population, and are associated with significant morbidity and increased mortality. It is now widely accepted that most autoimmune endocrine disorders have a genetic basis. Two rare autoimmune endocrine syndromes are associated with single gene mutations: (a) the APECED syndrome (autoimmune polyendocrinopathy, candidiasis, ectodermal dystrophy; also known as autoimmune polyendocrinopathy syndrome type 1) resulting from mutations in the *AIRE* gene on chromosome 21 and (b) the IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) caused by mutations in the *FOXP3* gene on the X chromosome (1, 2). In contrast, the majority of autoimmune endocrinopathies are inherited as complex genetic traits, with multiple genetic factors interacting with each other and with environmental factors to confer susceptibility to these disorders. With the exception of the major histocompatibility complex (MHC), these genetic factors remain largely unknown, although in recent years considerable efforts have been made towards their definition. Through these investigations, the cytotoxic T lymphocyte antigen-4 (*CTLA-4*) gene has emerged as an important susceptibility locus for autoimmune endocrinopathies.

CTLA-4, co-stimulation and T-cell activation

The *CTLA-4* gene, which is located on chromosome 2q33, encodes a co-stimulatory molecule that is expressed on the surface of activated T cells (3). The CTLA-4 molecule, together with CD28 (another co-stimulatory molecule expressed on the surface of both resting and activated T cells), plays a critical role in the T-cell response to antigen presentation. T-cell activation is initiated when the antigen-specific cell-surface T-cell receptor (TCR; CD3 complex) engages the antigen, which is bound to an MHC class II molecule on the surface of an antigen-presenting cell (Fig. 1). However, to complete this activation, leading to T-cell proliferation and cytokine production, a second signal (co-stimulatory signal) is required. In the absence of a positive co-stimulatory signal, the antigen–TCR engagement is ineffective, and causes the T cell to be refractory to further stimuli (anergy) or induces apoptosis of the cell. This positive co-stimulatory signal is provided mainly by the interaction of CD28 with its ligands, B7.1 (CD80) and B7.2 (CD86) on antigen-presenting cells. CTLA-4 also binds to the same B7 ligands (CD80 and CD86) but, in contrast to CD28, it delivers inhibitory signals to T-cell activation (4). However, CTLA-4 has a much greater affinity for B7 molecules than CD28. In the quiescent state, the majority of CTLA-4 is stored in intracellular vesicles. Upon TCR engagement and T-cell activation, these vesicles are

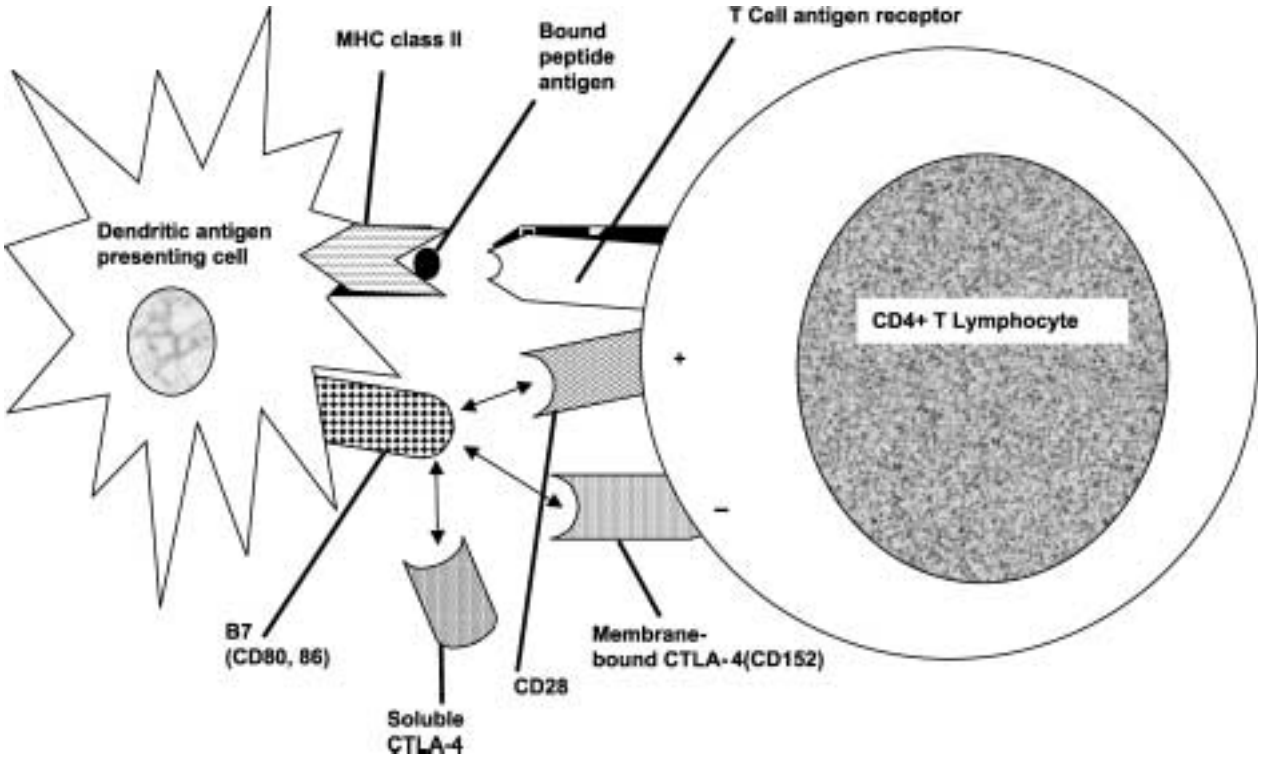

Figure 1 T-cell activation and co-stimulatory signals. T-cell activation requires two signals: the first signal is provided by antigen–TCR engagement and the second (co-stimulatory) signal is mainly provided by the interaction of the co-stimulatory molecule, CD28, with its ligands B7.1/B7.2, on antigen-presenting cells. CTLA-4, another co-stimulatory molecule also binds to B7.1/B7.2 but, in contrast to CD28, it provides a negative signal to T-cell activation. The altered levels of soluble CTLA-4 could lead to either blockade of available B7 molecules leading to a decreased stimulatory signal from CD28 engagement (if soluble CTLA-4 is increased), or to an inability of membrane-bound CTLA-4 to engage with B7s and hence less negative signalling (if soluble CTLA-4 levels are reduced). Further studies will help to clarify the mechanism.

rapidly mobilised to the cell surface, allowing expression of CTLA-4 and producing competition for CD28 in engaging the B7-binding sites. Recently, three novel co-stimulatory molecules have been identified: inducible co-stimulator (ICOS) providing positive, and programmed death-1 (PD-1) and B and T lymphocyte attenuator (BTLA) delivering negative co-stimulatory signals (5, 6).

Negative regulatory role of CTLA-4 on T-cell activation

Several lines of evidence have confirmed that CTLA-4 inhibits T-cell activation. CTLA-4 antibodies that were cross-linked with a second antibody or immobilised on beads have been found to prevent T-cell activation (4, 7). In murine models, the administration of CTLA-4-blocking antibodies augments anti-tumour immunity (8), enhances the immune response induced by mycobacterial, protozoal and nematode infections (9–11), exacerbates experimental allergic encephalitis (12) and precipitates the onset of diabetes in the TCR transgenic non-obese diabetic mouse (13). In addition, a soluble fusion protein of CTLA-4 and the immunoglobulin G1 Fc region prolongs the survival of transplant grafts (14) and ameliorates different experimental autoimmune disorders, including lupus (15) and diabetes (16). Furthermore, CTLA-4-deficient mice develop a rampant lymphoproliferative disorder resulting in splenomegaly, lymphadenopathy and death within 3–4 weeks of birth (17). These mice have a massive infiltration of autoreactive T cells into the parenchyma of several organs, leading to their destruction. Finally, the engagement of CTLA-4 with its ligands inhibits interleukin-2 production, arrests the progression of activation-induced T-cell cycling (18) and induces apoptosis of activated T cells (19, 20).

How CTLA-4 downregulates T-cell activation is not fully understood, although a number of putative mechanisms have been proposed. First, CTLA-4 may inhibit T-cell activity by competing with CD28 for the shared B7 ligands (21). Secondly, CTLA-4 may transduce negative signal through the TCR-associated protein kinases. On the T-cell surface, TCRs are associated with CD3 complex molecules (CD3γ, CD3δ, CD3ϵ, together with a disulfide-linked CD3ζ chain homodimer) that have tyrosine-based activation motifs in the cytoplasmic tails. Following TCR engagement in the presence of a co-stimulatory signal, these tyrosine motifs undergo phosphorylation by protein kinases

(Lck, Fyn and ZAP-70), which leads to signal transduction resulting in the nuclear transcription of genes involved in cellular proliferation and cytokine production. T cells from CTLA-4-deficient mice show constitutively activated TCR-associated protein kinases, Lck, Fyn and ZAP-70 as well as the Ras regulator $p52^{SHC}$, suggesting that CTLA-4 inhibits the TCR signalling pathway by interfering with the activities of these molecules (22). Finally, it has been demonstrated that CTLA-4 also modulates very early TCR signalling events by directly interacting with the CD3ζ chain of the TCR complex (23).

CTLA-4 and autoimmune thyroid diseases (AITD)

The inhibitory effect of CTLA-4 on T-cell activation has led to investigations into its role in different human autoimmune disorders. In 1995, Yanagawa *et al.* (24) first reported an association of GD with a 106 bp allele of the microsatellite polymorphism, *CTLA-4(AT)n* within the *CTLA-4* gene. Later, it was found that the G allele of the *CTLA-4* exon 1 single nucleotide polymorphism (SNP), *CTLA-4(49)A/G*, is also associated with GD (25). The association of these two polymorphisms of *CTLA-4* was replicated by several case-control studies (Table 1) and two family-based association studies (35, 51, reviewed in 52). In contrast, association studies of the promoter polymorphism *CTLA-4(-318)C/T* in GD showed less consistent results (Table 1). Moreover, it has been suggested that, in patients with GD, alleles of *CTLA-4* may influence the severity of thyrotoxicosis (35), the development of ophthalmopathy (53), the risk of relapse following anti-thyroid drugs (42) and the presence other co-existing autoimmune disorders (e.g. T1DM) (33). In keeping with these findings, Kouki *et al.* (54) showed an

Table 1 Case-control association studies of the *CTLA-4* polymorphisms in GD.

Marker and study	Population	Cases (*n*)/controls (*n*)	Odds ratio	*P* value
CTLA-4(AT)n				
Yanagawa *et al.* (24)	White (USA)	133/85	2.8	0.012
Kotsa *et al.* (26)	White (UK)	112/91	2.1	0.006
Sales *et al.* (27)	Japanese	31/97	NS	NS
Akamizu *et al.* (28)	Japanese	186/218	1.8	<0.01
Hadj Kacem *et al.* (29)	Tunisian	144/205	6.3	0.001*
CTLA-4(49)A/G				
Nisticò *et al.* (25)	Chinese (Hong Kong)	94/77	1.7	0.037
Donner *et al.* (30)	White (Germany, Canada)	305/325	1.6	<0.002
Marron *et al.* (31)	Chinese	28/94	NS	NS
Yanagawa *et al.* (32)	Japanese	153/200	1.6	<0.01
Awata *et al.* (33)	Japanese	112/425	1.4	0.049
Djilali-Saiah *et al.* (34)	White (France)	73/100	NS	NS
Heward *et al.* (35)	White (UK)	379/363	1.6	<0.0002
Buzzetti *et al.* (36)	White (Italy)	92/244	1.8	0.049
Chen *et al.* (37)	African American (US)	49/47	NS	NS
Park *et al.* (38)	Korean	97/199	1.6	0.034
Chistyakov *et al.* (39)	Russian	78/93	3.2	<0.001
Villanueva *et al.* (40)	White (US)	137/121	1.6	0.007
Allahabadia *et al.* (41)	White (UK)	484/424	1.8	<0.0001
Hadj Kacem *et al.* (29)	Tunisian	144/205	1.4	0.03**
Kinjo *et al.* (42)	Japanese	144/110	1.6	0.0095
Yung *et al.* (43)	Chinese (Hong Kong)	123/158	1.5	0.02
Kouki *et al.* (44)	White (USA)	120/80	2.7	<0.01
Vaidya *et al.* (45)	White (UK)	301/349	1.6	5.9×10^{-6}
Bednarczuk *et al.* (46)	White (Poland)	264/194	1.5	0.003
	Japanese	319/112	1.6	0.003
Mochizuki *et al.* (47)	Japanese	20/60	2.9	0.04
Kalantari *et al.* (48)	Iranian	90/90	1.6	0.025
CTLA-4(-318)C/T				
Braun *et al.* (49)	White (Germany, Canada)	125/173	2.2	0.006
Heward *et al.* (50)	White (UK)	188/355	NS	NS
	Chinese (Hong Kong)	98/82	NS	NS
Park *et al.* (38)	Korean	97/199	2.1	0.015
Hadj Kacem *et al.* (29)	Tunisian	144/205	NS	NS
Kouki *et al.* (44)	White (USA)	120/80	NS	NS
Vaidya *et al.* (45)	White (UK)	292/290	NS	NS

NS, not significant.
* Association with a rare allele (the 224 mobility unit (mu) allele; three in controls and 15 in Graves' patients).
** Association with the A allele (in contrast to the G allele as in other studies).

increased proliferation of stimulated T cells from subjects with GG genotypes at *CTLA-4(49)A/G*. Furthermore, we were able to show strong evidence of linkage of *CTLA-4* in GD sib-pairs from the UK, suggesting that this locus may confer up to one-third of the total genetic susceptibility to GD in this population (51). Apart from GD, the *CTLA-4* alleles have been found to be associated with AH (Table 2). In addition, linkage and association of *CTLA-4* with the presence of thyroid antibodies have also been reported (59, 60). Curiously, however, whole genome scans in AITD have failed to detect linkage at this locus (61–64).

CTLA-4, T1DM and AAD

In T1DM, linkage to the *CTLA-4* locus was first observed in a small cohort of Italian multiplex families (25). Subsequent transmission disequilibrium analyses (25, 31, 65, 66) and case-control studies (Table 3) have supported the role of *CTLA-4* in T1DM, although the association was not as consistent in T1DM as had been found in AITD. In case-control studies, the *CTLA-4* alleles were also associated with AAD (56, 85, 86), and this association appears to be stronger in patients with autoimmune polyendocrinopathy syndrome type 2 (AAD coexisting with T1DM and/or AITD) than in those with isolated AAD (86). As well as the autoimmune endocrinopathies, *CTLA-4* has been associated with a wide range of other autoimmune disorders, including primary biliary cirrhosis (87), multiple sclerosis (88), coeliac disease (89) and rheumatoid arthritis (90, 91). These observations have suggested that *CTLA-4* is a general autoimmune locus, and that the susceptibility polymorphism(s) within the gene may lead to general defects in the immune regulation, while other tissue-specific (e.g. insulin gene polymorphisms) or antigen-specific (e.g. MHC) genetic factors and environmental factors determine the involvement of particular target organs. Furthermore, the finding of common susceptibility loci for different autoimmune disorders, such as *CTLA-4*, may also explain the majority of the concordance of different autoimmune disorders in the same patients or their families.

Fine mapping study of *CTLA-4*

In a recent study, Ueda *et al.* (92) have performed a detailed genomic analysis of *CTLA-4* in three autoimmune endocrinopathies, GD, AH and T1DM. They sequenced the 300 kb region of chromosome 2q33 containing *CD28*, *CTLA-4* and *ICOS* genes to identify novel SNPs in the region, and subsequently genotyped a total of 108 SNPs and the *CTLA-4(AT)n* microsatellite polymorphism in nearly 400 patients with GD and controls for association studies. By constructing linkage disequilibrium 'blocks' and regression modelling, they were able to map the susceptibility locus to 6.1 kb region 3′ of the *CTLA-4* gene, containing four SNPs designated CT60, JO31, JO30 and JO27_1. Although the CT60 SNP was the most associated marker, they were unable to further dissect the susceptibility locus with respect to the four SNPs at the peak of association. The prevalence of the susceptibility allele (G allele) at the CT60 SNP was 63% in GD patients and 53% in healthy controls (odds risk (OR) 1.51). The same allele was also associated with AH (OR 1.45), and

Table 2 Case-control association studies of the *CTLA-4* polymorphisms in AH.

Marker and study	Population	Cases (*n*)/controls (*n*)	Odds ratio	*P* value
CTLA-4(AT)n				
Kotsa *et al.* (26)	White (UK)	44/91	2.2	0.02
Sale *et al.* (27)	Japanese	48/97	NS	NS*
Akamizu *et al.* (28)	Japanese	163/218	NS	NS
Petrone *et al.* (55)	White (Italy)	126/301	NS	NS
CTLA-4(49)A/G				
Donner *et al.* (56)	White (Germany, Canada)	73/466	1.6	<0.02
Awata *et al.* (33)	Japanese	88/425	1.5	0.029
Park *et al.* (38)	Korean	110/199	NS	NS
Petrone *et al.* (55)	White (Italy)	126/301	NS	NS
Nithiyananthan *et al.* (57)	White (UK)	155/376	1.6	0.001
Tomoyose *et al.* (58)	Japanese	143/199	1.8	<0.0001
CTLA-4(-318)C/T				
Heward *et al.* (50)	White (UK)	90/355	NS	NS
Braun *et al.* (49)	White (Germany, Canada)	64/173	NS	NS
Park *et al.* (38)	Korean	110/199	NS	NS
Tomoyose *et al.* (58)	Japanese	143/199	NS	NS

NS, not significant.
*Association found only in subgroups of idiopathic myxoedema patients with ($P = 0.01$) and without ($P = 0.004$) TSH-binding inhibitory immunoglobulin (TBII).

Table 3 Case-control association studies of the *CTLA-4* polymorphisms in T1DM.

Marker and study	Population	Cases (*n*)/controls (*n*)	Odds ratio	*P* value
CTLA-4(AT)n				
Lowe *et al.* (67)	White (Sweden)	606/502	1.84	0.002
Ihara *et al.* (68)	Japanese	160/200	0.54*	0.0012
Ban *et al.* (69)	Japanese	118/195	NS	NS
Graham *et al.* (70)	White (Sweden)	751/502	1.3	0.0001
CTLA-4(49)A/G				
Donner *et al.* (30)	White (Germany)	293/325	1.4	$<4\times10^{-3}$
Marron *et al.* (31)	White (USA)	244/274	NS	NS
	White (Spain)	89/57	NS	NS
	Korean	97/112	NS	NS
	Chinese	180/379	NS	NS
Van der Auwera *et al.* (71)	White (Belgium)	525/530	1.5	<0.005
Awata *et al.* (33)	Japanese	173/425	NS	NS**
Djilali-Saiah *et al.* (34)	White (France)	112/100	1.8	0.002
Krokowski *et al.* (72)	White (Poland)	192/136	1.7	0.002
Hayashi *et al.* (73)	Japanese	117/141	NS	NS***
Yanagawa *et al.* (74)	Japanese	110/200	NS	NS
Abe *et al.* (75)	Japanese	111/445	NS	NS****
Lee *et al.* (76)	Chinese (Taiwan)	253/91	1.7	0.0051
Kikuoka *et al.* (77)	Japanese	125/200	1.5	0.018
Ihara *et al.* (68)	Japanese	160/200	1.8	0.0002
Takara *et al.* (78)	Japanese	74/107	1.3	0.01
Klitz *et al.* (79)	Filipino	90/94	1.9	0.003
Ongagna *et al.* (80)	Alsacian	62/84	5.6	<0.05
Fafardy *et al.* (81)	White (France)	134/273	NS	NS
Cinek *et al.* (82)	White (Czech)	305/289	NS	NS
Ma *et al.* (83)	Chinese	31/36	3.6	<0.0005
Mochizuki *et al.* (47)	Japanese	97/60	NS	NS***
CTLA-4(-318)C/T				
Lee *et al.* (84)	Chinese (Taiwan)	347/260	1.9	0.0026
Ihara *et al.* (68)	Japanese	160/200	NS	NS

NS, not significant.
* Negative association found with the 86 bp allele.
** Association found in a subgroup of patients with insulin depletion and requiring insulin within 1 month of diagnosis ($P=0.012$).
*** Association found in a subgroup of patients with glutamic acid decarboxylase (GAD) antibodies ($P<0.05$).
**** Association found in a subgroup of patients with islet-specific islet cell antibody 512 antibodies ($P=0.004$).

less strongly with T1DM (OR 1.15). This high frequency of a low penetrance disease allele in the normal population could go some way to explaining why it has been difficult to confirm linkage in some genome scanning studies.

Functional studies showed that the associated haplotype at *CTLA-4* appeared to correlate with lower mRNA levels of a soluble form of CTLA-4 (sCTLA-4), which was determined by a splice variant missing the exon that encodes the transmembrane domain (92). In a rodent model of T1DM (the non-obese diabetic mouse), reduced production of a splice form of CTLA-4 that lacked the B7 ligand-binding domain was also found, adding further weight to the findings. Although Ueda and colleagues (92) studied CTLA-4 mRNA levels, an earlier investigation had found that elevated (rather than reduced) plasma levels of sCTLA-4 protein were more frequent in patients with AITD (11 out of 20) than in healthy controls (1 out of 30) (93). The finding of higher sCTLA-4 serum protein levels in subjects with GD has recently been confirmed (94). It remains unknown how these alterations of sCTLA-4 levels may predispose to the development of autoimmunity, although it has been speculated that a reduction in sCTLA-4 level could lead to reduced blocking of CD80/CD86 molecules, causing unopposed T-cell activation through CD28 (92). Conversely, higher levels of sCTLA-4 could compete with membrane-bound CTLA-4 for CD80/CD86-binding sites and cause a reduction of inhibitory signalling. Further work on the dynamics of membrane-bound versus sCTLA-4 expression and turnover are needed to resolve this issue.

Conclusions

There is now solid evidence for the role of *CTLA-4* in autoimmune endocrinopathies, including GD, AH and T1DM. With the success of the Ueda (92) study in fine mapping the *CTLA-4* locus, it can be envisaged that, in forthcoming years, more susceptibility genes for different complex traits will be identified through similar high-resolution genetic studies of candidate genes using large numbers of patients and controls. Moreover, it opens up the possibility that many other

complex traits may be determined by polymorphisms in regulatory sequences that control splice variants of a transcript, rather than by coding polymorphisms or those that encode purely quantitative differences in transcript expression.

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Received 19 November 2003
Accepted 6 February 2004