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Influence of CTLA-4 gene polymorphism in autoimmune and infectious diseases

Mercedes Fernández-Mestre [a], Karen Sánchez [a], Omar Balbás [a], Ketevan Gendzekhzadze [a,b], Violeta Ogando [a], Mayra Cabrera [c], Zulay Layrisse [a,*]

[a] Laboratorio de Fisiopatología, Centro de Medicina Experimental "Miguel Layrisse," Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela
[b] Department of Structural Biology, Stanford University School of Medicine, Sherman Fairchild Building, Stanford, CA, USA
[c] Instituto de Biomedicina, Universidad Central de Venezuela, Caracas, Venezuela

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ABSTRACT

Cell-mediated immunity requires costimulatory activity to initiate or inhibit antigen-specific T-cell responses. CTLA-4 is an inhibitory receptor expressed by activated and regulatory T cells. The single nucleotide polymorphism (SNP) +49 A/G of the *CTLA-4* gene alters intracellular distribution of CTLA-4, interleukin-2 production, and, as a consequence, T-cell proliferation. The aim of this study was to analyze the only coding SNP *CTLA-4* +49 A/G polymorphism in patients with either infectious (Chagas's, Dengue, and American cutaneous leishmaniasis) or autoimmune diseases (myasthenia gravis, pemphigus, and psoriasis). No statistically significant differences were reported when all patients of each disease group were compared with healthy individuals. However, the +49 G/G genotype was moderately increased in pemphigus and myasthenia gravis. Patients with diffuse cutaneous leishmaniasis (DCL) exhibited an increased frequency of the A/G +49 genotype compared with patients with localized cutaneous leishmaniasis (LCL; $p = 0.009$; odds ratio [OR] = 4.25; 95% confidence interval [CI] = 1.245–14.501) and intermediate cutaneous leishmaniasis (ICL; $p = 0.027$; OR = 4.44; 95% CI = 1.273–15.516), indicating that the heterozygous genotype, associated with overactivation of T-cell proliferation, could confer susceptibility to the development of the more severe clinical form of cutaneous leishmaniasis. The A/A +49 genotype was increased in LCL patients compared with DCL patients ($p = 0.019$; OR = 0.25; 95% CI = 0.067–0.953), indicating that this genotype, which has been associated with normal proliferation of T cells, could confer protection to the development of DCL. The results indicate that the polymorphism of CTLA-4 is an important genetic factor associated with risk or protection for the development of diffuse cutaneous leishmaniasis and has influence in the pathogenesis of autoimmune diseases. However, other closely linked candidate genes in linkage disequilibrium with CTLA4, such as CD28 and ICOS, could be associated with the development of autoimmune and infectious disease.

1. Introduction

CTLA-4 plays an essential role in immunologic homeostasis as a negative regulator of T-cell activation; however, little is known about the regulatory mechanism for CTLA-4 expression. This inhibitory signal not only determines whether T cells become activated, but also affects the clonal representation in an immune response [1]. In addition, CTLA-4 is highly expressed by T regulatory cells and could play an important role in their function [2]. Polymorphisms at certain locations of the *CTLA-4* might conceivably affect its expression [1]. About 16 single nucleotide polymorphisms (SNP) and a (AT)n microsatellite were reported at the *CTLA-4* locus [3]; however, only 3 of them (SNP +49, −318, and the microsatellite) have been widely studied. The SNP +49 A/G is located at position +49 in the first exon of the *CTLA-4* gene, leading to a threonine to alanine change in amino acid position 17 of the leader peptide. Recently, it has been reported that the +49 A/G polymorphism of the *CTLA-4* gene alters intracellular distribution of CTLA-4 and interleukin (IL)-2 production and, as a result, alters T-cell proliferation [4,5]. In humans, several reports have established an association between specific polymorphisms of the *CTLA-4* gene and autoimmune diseases. The potential explanation is that the CTLA-4:B7 interaction plays a critical role in regulating self-tolerance and hence susceptibility to autoimmune disease [6]. Likewise, in infectious diseases where T cells are fundamental components of the mechanisms of defense of the host versus pathogens, the polymorphism of the *CTLA-4* gene could play a role in the regulation of the immune response. We selected just one SNP for study because a direct correlation between the +49 A/G polymorphism of the *CTLA4* gene and T-cell function, as well as that between the *CTLA-4* genotype and the function of the CTLA-4 protein in T cells, has been reported. Therefore, CTLA-4 exerted less profound inhibitory effects on T-cell proliferation in patients bearing the G/G genotype than in patients bearing the A/A genotype. A *CTLA-4* gene polymorphism in the leader sequence (exon 1) may influence the level or the pattern of

* Corresponding author.
E-mail address: zlayriss@ivic.ve (Z. Layrisse).

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Table 1
CTLA-4 (SNP +49) genotype and allele frequency differences among patients with autoimmune and infectious diseases and control subjects

Genotype/allele	MG (n = 37)	PE (n = 48)	PS (n = 54)	D (n = 77)	CH (n = 105)	L (n = 69)	Control (n = 98)
AA	29.7 (11)	22.9 (11)	38.9 (21)	35.06 (27)	37.14 (39)	37.68 (26)	32.65 (32)
AG	46.0 (17)	43.7 (21)	48.1 (26)	45.45 (35)	47.61 (50)	44.92 (31)	47.95 (47)
GG	24.3 (9)	33.3 (16)	13.0 (7)	19.48 (15)	15.24 (16)	17.39 (12)	19.38 (19)
A	52.7 (39)	44.8 (43)	63.0 (68)	57.79 (89)	60.95 (128)	60.14 (83)	56.63 (111)
G	47.3 (35)	55.2 (53)	37.0 (40)	42.21 (65)	39.05 (82)	39.85 (55)	43.37 (85)

Entries are percentages and numbers (in parentheses) of individuals or chromosomes.
Organ-specific autoimmune diseases: MG = myasthenia gravis; PE = phemphigus; PS = psoriasis. Infectious diseases: D = Dengue; CH = Chagas's disease; L = leishmaniasis; CG = control group (healthy individuals).

expression of the protein. Alternatively, the trafficking properties of CTLA-4 may be altered, such that the total level of CTLA-4 may be similar but the CTLA-4 levels responsible for the negative signaling capacity of the molecule may be reduced. Differences in the leader sequence of the gene may result in altered rates of endocytosis or surface trafficking [4].

The aim of this study was to analyze the +49 A/G polymorphism of the *CTLA-4* gene in Venezuelan patients with organ-specific autoimmune diseases (myasthenia gravis [MG], pemphigus [PE], and psoriasis [PS]) and patients with infectious diseases (Chagas's [CH], dengue [D], and American cutaneous leishmaniasis [L]).

2. Subjects and methods

2.1. Patient and control populations

Whole blood was collected from 405 patients grouped in two series: patients with either infectious or autoimmune diseases. Patients with autoimmune diseases included 150 individuals with three organ-specific autoimmune diseases: 46 with MG, 50 with PE, and 54 with PS.

PE presented in two clinical forms: pemphigus foliaceus (PF) and pemphigus vulgaris (PV). The numbers of patients with infectious diseases were as follows: 77 with D, 105 with CH, and 73 with L. The individuals with D viral infection were classified using all available clinical and laboratory data [7] into two different clinical groups: patients with dengue fever (DF; n = 45) and patients with dengue hemorrhagic fever (DHF; n = 28). Four patients, although they were infected with D, were not clinically classified. The patients with CH were classified according to clinical and electrocardiograph (ECG) characteristics [8] as asymptomatic or patients without cardiac symptoms (Group A, n = 34), patients with arrhythmia-related symptoms (Group B, n = 38), and patients with overt congestive heart failure (Group C, n = 35). The individuals infected with *Leishmania* parasite were classified into three groups according to clinical, histological, and immunological characteristics [9]: localized cutaneous leishmaniasis (LCL, n = 27); intermediate cutaneous leishmaniasis (ICL; n = 25), and diffuse cutaneous leishmaniasis (DCL; n = 17).

All patients were ethnically mixed Venezuelans (i.e., born in Venezuela and descended from mainly Spanish Europeans, autochthonous inhabitants, and West Africans).

Ninety-eight unrelated healthy subjects of similar ethnic background were tested as controls.

A local ethics committee approved the research protocol.

2.2. CTLA-4 SNP +49 typing

Genomic DNA was extracted from blood samples using a modified salting-out procedure [10]. Polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) was used to establish the *CTLA-4* genotypes as previously described [4,11,12], with some modifications. Briefly, two pairs of primers were used to amplify the appropriate segment of the *CTLA-4* gene containing SNP +49. The PCR product was incubated with BbvI (Gibco, Rockville, MD) for 16 hours at 37°C and analyzed on 4% agarose gels.

2.3. Statistical analysis

Allele, genotype, and haplotype frequencies were determined by direct counting. The statistical significance of allele frequency differences between patients and controls was estimated using the Mantel—Haenszel χ^2 test (because of the small sample size) using 2×2 contingency tables; p values were corrected by multiplying the number of comparisons made (Bonferroni correction) and considered significant when $p < 0.05$ [13]. Relative risks with corresponding 95% confidence intervals (95% CI) were calculated as odds ratios (OR) according to Woolf's formula [14] or by the modified method described by Haldane [15] when one element of the equation was zero.

3. Results

3.1. The SNP +49 (A/G) of the CTLA-4 gene in two series of patients and healthy individuals

The distribution of *CTLA-4* genotype frequencies in control subjects and patients with infectious and autoimmune diseases was in Hardy—Weinberg equilibrium (Table 1). No statistically significant differences were reported when the control group was compared with all patients in each series. The A/G +49 genotype was the most common genotype in every group (healthy controls and patient groups). However, the frequency of the +49A allele was increased among patients with infectious diseases (D + CH + L) compared with patients with autoimmune diseases (MG + PE). Although PS is considered an autoimmune disease, the *CTLA-4* genotype frequencies in these patients was not very different from the frequencies observed in patients with infectious diseases.

3.2. The SNP +49 (A/G) of the CTLA-4 gene in patients with infectious or autoimmune diseases grouped clinically

The distribution of *CTLA-4* genotype frequencies in the different groups of patients with L exhibited an increased frequency of the A/G +49 genotype in patients with DCL compared with patients with LCL (71.42% vs. 37.03%; p = 0.009; OR = 4.25; 95% CI = 1.245–14.501) and ICL (71.42% vs. 36%; p = 0.027; OR = 4.44; 95% CI = 1.273–15.516). The A/A +49 genotype frequency was increased in patients with LCL compared with patients with DCL (48.14% vs. 19.04%; p = 0.019; OR = 0.25; 95% CI = 0.067–0.953) (Table 2). No statistically significant differences existed between patients with DF and patients with DHF. However, the frequency of the A/A +49 genotype associated with normal expression levels of CTLA-4 is moderately decreased in DHF patients compared with DF patients (28.57% vs. 40.9%).The distribution of *CTLA-4* genotype frequencies in the different groups of serologically positive for *Trypanosoma cruzi* patients, (asymptomatic, arrhythmic, and cardiomyopathic) demonstrated no association between the *CTLA-4* (SNP +49) polymorphism and different clinical forms of CH.

Finally, both groups of patients with PE exhibited a similar frequency of the A/G +49 genotype; however, the frequency of the A/A +49 genotype was increased in patients with PF compared with patients with PV (36.36% vs. 20.51%; OR = 2.21; 95% CI =

Table 2
CTLA-4 (+49) genotype and allele frequencies in three clinical groups of Leishmania infection (LCL, ICL, and DCL)

Genotype/allele	LCL ($n = 27$)	ICL ($n = 25$)	DCL ($n = 17$)
AA	48.14 (13)	40 (10)	17.64 (3)
AG	37.03 (10)	36 (9)	70.58 (12)
GG	14.81 (4)	24 (6)	11.76 (2)
A	66.66 (36)	58 (29)	52.94 (18)
G	33.33 (18)	42 (21)	47.05 (16)

Entries are percentages and numbers (in parentheses) of individuals or chromosomes. LCL = localized cutaneous leishmaniasis; ICL = intermediate cutaneous leishmaniasis; DCL = diffuse cutaneous leishmaniasis.

0.517–9.473; p = n.s.). In addition, when *CTLA-4* genotype frequencies among patients with PV versus healthy subjects were compared, an increased frequency of G/G +49 genotype was observed in PV patients compared with healthy individuals (37.83% vs. 19.38%; $p = 0.021$; OR = 2.32; 95% CI = 1.021–5. 307). Likewise, in PV patients the +49 G allele was increased (OR = 1.78; 95% CI = 1.047–3.027; $p = 0.016$) compared with the control group (Table 3). No statistically significant differences existed when the control group was compared with the group of patients with MG and those with psoriasis PS.

4. Discussion

The +49 A/G polymorphism of the CTLA-4 gene alters intracellular distribution of CTLA-4 and IL-2 production, influencing T-cell proliferation [4,5]. The CTLA-4 gene yields at least two major mRNA transcripts in humans. One transcript encodes a transmembrane protein and the other encodes a soluble form of CTLA-4 that lacks a transmembrane domain. At the protein level, a variety of studies have implicated elevated levels of the sCTLA-4 protein in the plasma of patients with a variety of immunologically mediated diseases. Recently, Berry *et al.* studied four commonly tested SNPs within the *CTLA-4* region among 57 patients with autoimmune diseases and 27 normal volunteers with positive or negative (undetected) sCTLA-4 levels and reported no association between circulating levels of sCTLA-4 and SNP genotypes [16].

Table 3
CTLA-4 (SNP +49) genotype and allele frequencies with significant differences among diverse groups

CTLA-4 (SNP +49)	PV ($n = 37$)	CG ($n = 98$)	OR 95% IC	p
Genotypes				
GG	37.83 (04) ↑	19.38 (19)	2.53	0.022
Alleles				
G	59.45 (44) ↑	43.37 (85)	1.92	0.013
A	40.50 (30)	56.63 (111) ↑	0.522	0.013
	PV + MG ($n = 85$)	D + CH + L ($n = 251$)	OR 95% IC	p
Alleles				
A	48.23 (82)	59.76 (300) ↑	0.62	0.005
G	51.76 (88) ↑	40.23 (202)	1.59	0.005
	LCL ($n = 27$)	DCL ($n = 17$)	OR 95% IC	p
Genotypes				
AA	48.14 (13) ↑	17.64 (03)	0.23	0.04
AG	37.03 (10)	70.58 (12) ↑	4.08	0.03
	ICL ($n = 17$)	DCL ($n = 17$)	OR 95% IC	p
Genotypes				
AG	36 (09)	70.58 (12) ↑	4.26	0.028

Entries are percentages and numbers (in parentheses) of individuals or chromosomes. Organ-specific autoimmune diseases: MG = myasthenia gravis; PE = phemphigus; CG = control group (healthy individuals). Infectious diseases: D = Dengue; CH = Chagas's disease; L = leishmaniasis.

Our study of the (A/G) polymorphism of the *CTLA-4* gene in two series of patients, those with autoimmune and infectious diseases, indicated no significant genotype frequency difference when all patients in each group were compared with healthy individuals. However the +49 G/G genotype was moderately increased in patients with PE and MG. This genotype is associated with a reduced control of T-cell activation and in this way probably contributes to the pathogenesis in autoimmune diseases. Accumulating evidence verifies the importance of CTLA-4 in the pathogenesis of MG. Anti-CTLA-4 antibody treatment triggers determinant spreading and enhances murine MG [17]. Several studies have confirmed that *CTLA-4* polymorphism is associated with MG [18–20]. Recently, Pavoni *et al.* studied the influence of *CTLA4* −318 and +49 polymorphisms in the pathogenesis of PF; however, no statistically significant association between PF and variants of the *CTLA4* gene was observed [21], similar to our results. On the other hand, in our study the frequency of the G/G +49 genotype, associated with a reduced control of T-cell activation and in this way probably contributing to the pathogenesis, was increased in PV patients compared with healthy individuals. The potential explanation for the association between specific genetic polymorphisms of the *CTLA-4* gene and autoimmune diseases is that such genetic polymorphism may affect the expression or function of the CTLA receptor, thus resulting in autoreactivity [22].

Different mechanisms of action of CTLA-4 have been reported. One is delivery of a negative signal to T cells, with rapid inhibition of T-cell activation, and another is B7 (CD80, CD86) sequestration on antigen-presenting cells and induction of T-cell anergy [23]. A role for CTLA-4 in autoimmune disease is suggested by the observation that blockade of the B7:CTLA-4 interaction via administration of antibodies against CTLA-4 exacerbates autoimmune diseases in animal models, whereas stimulation via CTLA-4Ig ameliorates experimental autoimmune MG [reviewed in 20]; indeed, an exogenous recombinant CTLA-4/Ig molecule infused to the patients ameliorated symptoms in several autoimmune diseases including PS [24,25]. On the other hand, sCTLA-4 may similarly inhibit the interaction of the cell membrane-anchored CTLA-4 molecule with CD80 and CD86, thus blocking the inhibition of T-cell activation [20]. In light of the findings mentioned above, it has been hypothesized that elevated sCTLA-4 concentration in PS results in its successful competition with activated T-cell membrane-anchored CTLA-4 for interaction with CD80/CD86 ligands, weakening the control over T-cell response and resulting in the aggravation of disease symptoms [26]. Although in our study *CTLA-4* exon 1 polymorphism was not associated with susceptibility to PS, other variants such as T/C-1772, associated with levels of sCTLA-4 in sera [27], could play an important role in this autoimmune disease.

Our data indicate that the +49A allele increases the risk of development of viral and parasitic diseases (D, CH, L), but confers resistance to autoimmune diseases (MG, PE). In contrast, the +49 G allele might be a risk factor for autoimmune disease (PE). Different mechanisms are postulated to explain the protective effect of infections on autoimmune diseases, such as competition, regulation, and stimulation of innate immunity [28]. The occurrence of overdominant selection in the population (a heterozygote advantage), in which homozygotes for a specific allele are resistant to one disease but susceptible to another, could explain genetically the inverse relationship between infectious and autoimmune diseases [29]. There could be opposing effects of the two homozygotes: while one of the three genotypes confers susceptibility to one type of disease (infectious disease), the other confers susceptibility to a different disease (autoimmunity).

The analysis among the different clinical groups of patients infected with *Leishmania* demonstrated an increased frequency of the A/G +49 genotype in patients with DCL compared with patients with LCL and ICL, indicating that the heterozygous genotype, asso-

ciated with overactivation of T-cell proliferation, could confer susceptibility to the development of the more severe clinical form of cutaneous L. The A/A +49 genotype was increased in LCL patients compared with DCL patients, indicating that this genotype, which is associated with the normal proliferation of T cells, could confer protection to the development of DCL. Recently, Favali *et al.* demonstrated that CD28:B7 is important in IFN-γ and TNF-α production by peripheral blood mononuclear cells from cutaneous L patients [30]. These cytokines are essential to control parasite multiplication because they influence macrophage activation, and the blockade of the CD28:B7 pathway leads to downmodulation of both proinflamatory cytokines. The three active forms of American tegumentary L are characterized by cytokine patterns in lesions: LCL by a Th1 response, DCL by a Th2 response, and ICL by a mixed pattern of cytokines [31]. CTLA-4 engagement during differentiation inhibits polarization of naive $CD4^+$ cells to the Th2 but not the Th1 cell subset. Once polarized, CTLA-4 engagement inhibits cytokine production in both Th2 and Th1 effector cells. All together, these data indicate that CTLA-4 may interfere not only with the signaling involved in transcriptional activation of both Th1 and Th2 cells, but also with the development of one of the Th cell subsets [32]. CTLA-4 is a critical and potent inhibitor of Th2 differentiation. Thus, the B7-CD28/CTLA-4 pathway plays a critical role in regulating Th2 differentiation in two ways: CD28 promotes Th2 differentiation, whereas CTLA-4 limits Th2 differentiation. Hence, reductions in CTLA-4 expression (GG or A/G +49 genotype) promote a Th2 response that favors the development of DCL. Although DCL may be Th2 mediated and less effective, CTLA-4 could play a role (+49 G). MG is also antibody mediated, yet there is no association with +49 G, possibly because only a subgroup of MG/thymoma patients has been associated with CTLA-4 gene polymorphism. Our group included patients with thymic hyperplasia and patients without thymoma or hyperplasia; only 4/46 patients had thymoma.

The results indicate that the polymorphism of *CTLA-4* is an important genetic factor associated with the risk of or protection for development of diffuse cutaneous L, which is infrequent in Venezuela, with only 20 cases reported up to 1995 [33]. However, other closely linked candidate genes in linkage disequilibrium with *CTLA4*, such as *CD28* and *ICOS*, could be associated with the development of autoimmune and infectious disease.

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