

CD 28 gene polymorphism and plasma concentration of soluble CD 28 in Iranian patients with visceral leishmaniasis

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Abstract. In visceral leishmaniasis (VL), the development of protective immunity is associated with expansion of leishmania-specific T-cell responses. Because of the essential role of CD28 in the effectiveness of T-cell activation, this study was carried out to investigate the CD28 gene polymorphism and plasma levels of soluble (s) CD28 molecule in Iranian patients with VL. Plasma concentrations of CD28 in 88 patients with VL, 132 individual with subclinical leishmaniasis, and 100 seronegative healthy controls were measured by enzyme-linked immunosorbent assay. Genotyping of CD28 gene polymorphism was performed by polymerase chain reaction based allotyping method using allele-specific primers for C or T at intron 3 position +17 in three groups. The frequency of CC genotype was significantly higher in subclinical VL patients (42.4%) than active VL group (27.3%) and healthy controls (16%) ($P < 0.001$). Also, the frequency of allele C among subclinical VL group (57.6%) was significantly higher than active VL (40.9%) and control groups (34%) ($p = 0.003$). No significant differences were observed between the plasma levels of sCD28 in three groups. Our findings suggest that the CD28 gene may have significant role in the protection of active VL in the Iranian population.

INTRODUCTION

Visceral leishmaniasis (VL) is a parasitic disease with a wide range of clinical symptoms. The outcome of leishmania infection and the manifestation of disease depend on genetically determined human immune responses and environmental factors (Jeronimo *et al.*, 2005). The resolution of infection and the development of protective immunity are associated with expansion of leishmania-specific T-cell responses (Awasthi *et al.*, 2004).

It has been demonstrated that CD28 plays an essential role in the effectiveness of T-cell immune responses. CD28 is

constitutively expressed on almost all human CD4⁺ T cells and on ~50% of CD8 T cells and, as a major co-stimulatory molecule, binds to CD80/CD86 ligand on antigen presenting cells and delivers a potent co-stimulatory signal to T-cells (Lenshow *et al.*, 1996; Berg & Zavazava, 2008). Recent studies indicate that CD28 gene polymorphism is associated with some autoimmune and infectious diseases (Soriano *et al.*, 2002; Teutsch *et al.*, 2004).

In this study we investigated the CD28 gene polymorphism and plasma levels of sCD28 molecule in Iranian patients with visceral leishmaniasis.

MATERIALS AND METHODS

Subjects

During a 2-year period (from April 2005 to February 2006), 88 patients with VL (50% male, mean age: 7.9 ± 3.8 years), and 132 individuals with subclinical *Leishmania* infection (52% male, mean age: 9.1 ± 5.6 years) were enrolled in the study. The control group consisted of 100 ethnically matched seronegative healthy volunteers (50% male, mean age: 10.4 ± 3.3 years). All subjects were Iranian Caucasian from villages of Azerbaijan, an endemic area in north-west Iran.

Diagnosis of VL was established in all patients with a clinical suspicion of VL by demonstration of Leishman bodies in bone marrow aspirates and/or the presence of specific antibody titres more than 1/320 by Enzyme Linked Immunosorbent Assay (ELISA). Subclinical *Leishmania* infection was defined as asymptomatic individuals with ELISA titres of more than 1/320, but with no history of VL. Healthy controls were seronegative individuals with no history of VL.

For each individual enrolled in the study, a plasma sample was obtained and was stored at -20°C until use. Also, a sample of venous blood was collected in EDTA. DNA was extracted from blood by the salt-out technique, and stored at a final concentration of 200 $\mu\text{g}/\text{mL}$ until genotyping. Serum levels of sCD28 were measured by ELISA using reagent kits for human sCD28 (Bender Medsystems Diagnostics, Vienna, Austria).

CD28 genotyping

Genotyping was performed using a polymerase chain reaction (PCR) based allotyping method with allele-specific primers as described previously by Meyer *et al.* (2005). The primers used to detect T and C alleles were 5'-ATTTTCTGGGTAA GAGAAGCAGCACT-3' (T primer) and 5'-ATTTTCTGGGTAA GAGAAGCAGCACC-3' (C primer) respectively and the common primer 5'-ACCTACTCAATGCCTTCTGG

AAATC-3'. Internal control primers were 5'-GCCTTCCCAACCATTCCCTT-3' and 5'-TCACGGATTTCTGTTGTGT-TTC-3', and yielded a product of 221 bp. PCR products were visualized by electrophoresis in agarose gel.

The Ethical Committee of Research Center for Infectious Diseases, Tabriz University of Medical Sciences, approved the study protocol.

Statistical analysis

Data were analyzed using SPSS statistical package version 14, and a $P < 0.05$ was considered statistically significant. The mean sCD28 levels were compared by ANOVA test. To assess distribution differences of alleles and genotypes among VL, subclinical VL, and healthy control groups; we compared likelihood ratio of all three groups using Chi-Square test with 3×2 tables.

RESULTS

During the study period, a total of 88 patients with VL (44 males and 44 females) were included. The mean age of patients was 7.9 ± 3.8 years (Range: 2 -16 years). The most common clinical findings of VL were fever (100%) and splenomegaly (95%).

Table 1 shows the data of plasma sCD28 concentrations in study groups. No significant differences were found in the mean sCD28 concentration among patients with VL, those with subclinical *Leishmania* infection and healthy subjects.

The distributions of genotype and allele frequencies of the CD28 IVS3+17TC polymorphism are shown in Table 2. The frequency of CC genotype was significantly higher in subclinical VL group (42.4%) than active VL patients (27.3%) and healthy controls (16%) ($P < 0.001$).

Also, the frequency of the alleles among subclinical VL group (57.6%) was significantly higher than active VL group (40.9%) and healthy controls (34%) ($p = 0.03$).

Table 1. Plasma sCD28 concentrations of patients with visceral leishmaniasis, subclinical *Leishmania* infection, and healthy controls

Study Population	Mean \pm SD	Confidence interval
Visceral leishmaniasis	0.4 \pm 0.3	0.33 – 0.47
Subclinical infection	0.5 \pm 0.3	0.45 – 0.57
Control	0.5 \pm 0.3	0.46 – 0.61

Table 2. Genotype and allele frequencies of CD28 in study groups

	VL N = 88	Subclinical VL N = 132	Controls N = 100	P value
Genotype n(%):				
TT	40 (45.4)	36 (27.3)	48(48)	P = 0.003
CT	24 (27.3)	40 (30.3)	36(36)	NS ^a
CC	24 (27.3)	56 (42.4)	16 (16)	P < 0.001
Allele n (%):				
C	72 (40.9)	152 (57.6)	68 (34)	P = 0.003
T	104 (59.1)	112 (42.4)	132 (66)	NS

^aNS: Not significant

DISCUSSION

Visceral leishmaniasis is an endemic zoonosis in Iran, caused by *Leishmania infantum*. It predominantly affects children and may result in a wide range of clinical outcomes, from subclinical infection to fatal disease (Soleimanzadeh *et al.*, 1993; Davies & Mazloumi Gavvani, 1999). The outcome of leishmanial infection and the manifestations of disease depend on genetically determined human immune responses and environmental factors. There is evidence of both protective and disease-enhancing elements. Cytokines and chemokines play key roles in mediating the outcome of infection (Jeronimo *et al.*, 2005). In contrast to the earlier ideas that antagonistic functions of IFN- and IL-4 determine the outcome of protection or pathogenesis of the disease, recent studies emphasize the importance of the balance

of the two regulatory cytokines IL-12 and IL-10, critical for the regulation of the immune modulation during infection (Saha *et al.*, 2006). Further studies prove that this counter-regulatory activity of IL-10 and IL-12 plays a fundamental role in modulating the immune response of *Leishmania* infection in human towards Th1 or Th2 probably by modulating the B7/CD28 co-stimulatory interaction respectively. Nevertheless, despite extensive studies in murine models and in naturally infected humans, the precise consequence of events that determines the outcome of infection has not been fully elucidated (Saha *et al.*, 2006; Tripathi *et al.*, 2007).

Previous studies have demonstrated the increased cell surface expression and serum soluble concentration of T cell co-stimulatory molecules CTLA-4, CD28, CD80 and CD86 in patients with lupus erythematosus, rheumatoid arthritis,

primary Sjogren's syndrome, systemic sclerosis, and allergic asthma (Hebbar *et al.*, 2004; Hamzaoui *et al.*, 2005; Ip *et al.*, 2005; Wong *et al.*, 2005a; 2005b). There were also a few reports concerning the role of T cell co-stimulatory molecules in resistance to intracellular pathogens including *Mycobacterium tuberculosis* and *Toxoplasma gondii* (Villegas *et al.*, 2002; Rajavelu & Dos 2008). Animal studies have identified a critical role for CD28 in the memory responses required for resistance to *T. gondii* (Hunter *et al.*, 2003). Increased serum levels of sCD26 and sCD30 have been reported in patients with *L. infantum* infection (Ajdayr *et al.*, 2006).

It is known that the interaction of B7.1 and B7.2 co-stimulatory molecule, with CD28 is required for activation of T cells. It was reported that *Leishmania donovani* infection down regulated the B7.1 expression making it unable to deliver the co-stimulatory signal required for T helper cells differentiation, suggesting the regulatory role of co-stimulatory molecule in parasitic infection (Awasthi *et al.*, 2004). CD40 ligand (CD40L) present on the surface activated T cells interacts with CD40 on the macrophages and induces IL-12 expression and production. CD40-CD40L interaction helps in co-stimulation (Sugaya *et al.*, 2005; Stax *et al.*, 2008). A recent study showed that CD40L is required for control of *L. donovani* infection (Murray *et al.*, 2003). It has been also reported that the CTLA-4 is an inhibitory receptor which limits CD28-B7 co-stimulation (Murphy *et al.*, 1998), and anti-CTLA-4 monoclonal antibody is active as immunotherapy against VL (Hodi, 2007).

Apart from these findings, recent studies have demonstrated conflicting results about the association between the CD28 gene polymorphism and autoimmune disorders including systemic lupus erythematosus, multiple sclerosis, Behcet's disease, autoimmune hepatitis, type 1 diabetes, and autoimmune thyroid disease in different populations (Ahmed *et al.*, 2001; Djilali-Sajah *et al.*, 2001; Ihara *et al.*, 2001; Tomer *et al.*, 2001; Veen *et al.*, 2003; Gunesacar *et al.*, 2007). Other investigations

showed that the polymorphisms of T-cell regulatory genes (CD28 and CTLA-4) confer susceptibility to lymphoma and B-cell chronic lymphocytic leukemia (Cheng *et al.*, 2006; Suwalska *et al.*, 2008). However, only a few studies have been devoted to the role of CD28 polymorphism in infectious diseases (Malek *et al.*, 2004).

In the present study, we observed no association between sCD28 concentration and VL. However, our finding on the increased IVS3+17CC genotype frequency of the CD28 gene in individuals with subclinical VL suggest that CD28 plays an important role in activation of Th1 cells which result in protection against clinical VL. Compared to our findings, a previous study in our region reported increased serum levels of co-stimulatory molecule CD30 in VL. The explanation may be that the co-stimulatory molecules CD28 and CD30 are members of two distinct superfamilies. CD30 is a member of TNF superfamily which had a more complex cytoplasmic tail (Boesteanu *et al.*, 2009). The CD30 cell expression and secretion are dependent to factors distinct from those of CD28.

A question remains why there are no increased levels of sCD28 in spite of the role of CD28 gene polymorphism in outcome of VL. The appearance of this discrepancy may be explained by a number of factors. First, expression of CD28 on activated T cells may increase without raising serum levels of sCD28. Similar discrepancies have described for CD30 expressing cells and sCD30 in atopic allergic diseases (Esnault *et al.*, 1996). Expression of CD28 is essential in promoting the expansion of specific T cells particularly the Th1 cells which confer protection in VL infection (Jeronimo *et al.*, 2005; Rajavelu *et al.*, 2008). Second, the function of CD28 can be largely influenced by the presence of the other members of the CD28 family. CTLA-4 and ICOS are both structural homolog of CD28, yet they exhibit unique functions upon stimulation. CTLA-4 competes with CD28 for bindings to same ligands (B7-1 and B7-2), and it is not expressed on resting or newly activated

T cells. Binding of CTLA-4 by B7-1 or B7-2 inhibits T cell proliferation. In contrast to CTLA-4, the inducible co-stimulator ICOS, does not share ligands with CD28 but rather binds its own ligand B7-h and augments proliferation, antibody response and cytokine production (Boesteanu *et al.*, 2009). The sCD28 production may be implicated by CTLA-4 and ICOS functions.

Third, CD28 gene polymorphism may not affect the serum level of sCD28. Similar discrepancies have reported for other members of the CD28 family. Lack of association between sCTLA-4 levels in plasma and common CTLA-4 polymorphisms has been reported in immune disorders (Berry *et al.*, 2008, Purohit *et al.*, 2005).

In conclusion, the precise mechanisms that control levels of the sCD28 need to be further investigated. Our study is the first in the literature to report that gene polymorphism of CD28 contributes to resistance to VL. Further study is needed to ascertain the mechanisms of the relationship between CD28 and leishmaniasis.

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