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Evaluation of CTLA-4, CD28 and CD86 Genes Polymorphisms in Acute Renal Allograft Rejection among Tunisian Patients

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1. Introduction

Kidney transplantation is the preferred therapy for most patients with end-stage renal disease (Sui et al., 2008; Turgeon et al., 2009). Transplantation improves both quality of life and survival (Chavez et al., 2008). Unfortunately, the rate of renal allograft rejection remains important. With the aim of reducing the level of transplantation failure, several researches were achieved to elucidate the immunological mechanisms involved in this process. Thus, it was well established that allograft rejection is an immune response strongly depending on T cells proliferation. In fact, T lymphocytes play a crucial role in the initiation and the regulation of the adaptive immune response to foreign or native antigen (Vincenti, 2008). Herein, we focused on the costimulatory molecules: CTLA-4 and CD28: two receptors of T lymphocytes, and CD86: their common ligand on the antigen presenting cells (APC).

2. Costimulatory molecules pathway of t lymphocytes activation

The activation of naïve T cells requires two distinct signals. The first one is mediated by the association of the T cell receptor (TCR) with the Major Histocompatibility Complex (MCH) molecules, known in humans as the Human Leukocyte Antigen (HLA) (Thomas, 2007) and expressed on the antigen presenting cells (APCs). This interaction mediates the specificity of a T cell response by the recognition of specific epitopes of the presented antigen. The second signal is provided by the interaction between CD28 on the T cell surface and CD86 (B7-1) or CD80 (B7-2) on the APCs (Alegre et al., 2001; Handa et al., 2005). This co-stimulatory signal leads to clonal-T lymphocytes expansion and differentiation and to cytokines expression

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(Melichar et al., 2000; Seliger et al., 2008). In fact, the ligation of CD28 and CD3 (a T cell co-receptor) promotes the production of interleukins IL-4 and IL-5 and seems to be unique in its ability to induce very high levels of IL-2 production by prolonging the nuclear residency of nuclear factor of activated T cells (NFAT). These interleukins enhance T-helper-cell differentiation. CD28 engagement also confers critical survival signals to T cells which provides resistance to apoptosis and induces long-term expansion of T-cells (Beier et al., 2007; Wang et al., 2004) (Figure 1).

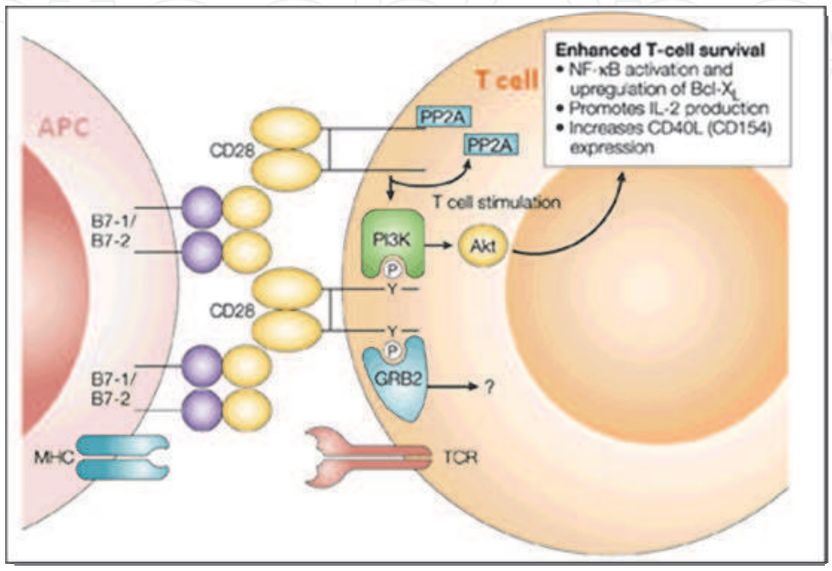


Fig. 1. Engagement of CD28 (Sharp, 2002). Signaling through CD28 promotes cytokine (IL-2) mRNA production and entry into the cell cycle, T-cell survival (at least in part by induction of Bcl-X_L), T-helper-cell differentiation and immunoglobulin isotype switching. (NF-kB, nuclear factor kappa b; PI3K, phosphatidylinositol 3-kinase; PP2A, protein phophatase 2A)

Following T cell activation, the expression on the T lymphocytes surface of the Cytotoxic T Lymphocyte Antigen-4 (CTLA-4), the counter receptor of the CD28, is rapidly up-regulated. CTLA-4 is the higher affinity receptor for both CD80 and CD86. The binding of CTLA-4 to these ligands down-regulates T lymphocytes proliferation (Beier, 2007; Collins, 2002; Wang, 2004). In fact, the engagement of CTLA-4 delivers negative signals, which inhibit IL-2 mRNA production, cytokines synthesis, cell cycle progression and then terminate T cell responses (Sharp, 2002) (Figure 2).

Several mechanisms by which CTLA-4 down-regulates T cells activation have been proposed. Thus, CTLA-4 might successfully compete with CD28 for CD80/86 and thereby inhibit the costimulatory effect of CD28. CTLA-4 could prevent constitutive expression of downstream signaling pathways by interacting with the protein tyrosine kinases (PTKs) Lck, Fyn and ZAP-70 through SHP1 phosphatase; now these PTKs play a key role in the TCR signaling. CTLA-4 might also directly interact with the TCR–CD3 complex at the immunological synapse to disrupt T-cell activation by binding and blocking the immunoreceptor tyrosine-based activation motif (ITAM) which serves as substrate to the PTKs, and, when its tyrosine residue is phosphorylated, induces the activation of ZAP-70 (Brand, 2005; Chuang et al., 1999) (Figure 3).

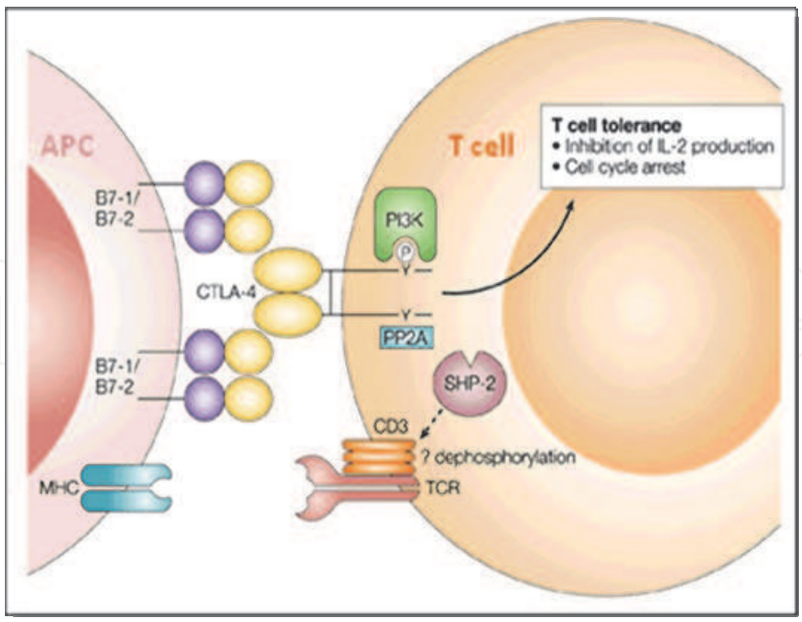


Fig. 2. Engagement of CTLA-4 (Sharp, 2002). Each CTLA-4 dimer can bind two independent B7-1/B7-2 homodimers. The crystal structure of B7-1/B7-2–CTLA-4 indicates that a linear zipper-like structure might form between B7-1/B7-2 and CTLA-4 homodimers.

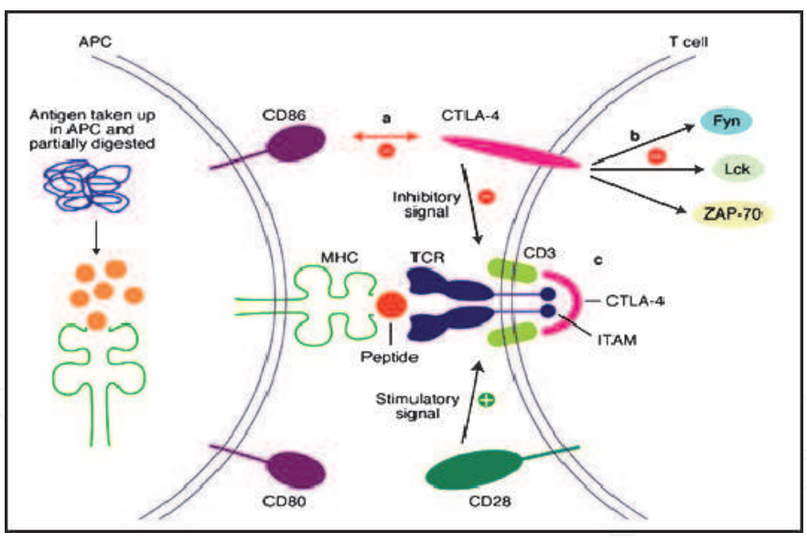


Fig. 3. Proposed mechanisms by which CTLA-4 inhibits T-cell activation (Brand, 2005)

Such down-regulation of T lymphocytes proliferation may induce immune tolerance, which is fundamental for allograft acceptance. Thus, the outcome of an immune response involves a balance between CD28-mediated T cell activation and CTLA-4-mediated inhibition.
Previous studies also focused on the costimulatory pathway of T lymphocytes activation. For instance, Minguela et al. analyzed the expression of costimulatory molecules in liver transplant and demonstrated that up-regulation of CD28/CTLA-4/CD86 molecules is associated with acute rejection of liver allograft (Minguela et al., 2000). Furthermore, it has

been demonstrated that the blockade of CD28-CD86 binding prolongs graft survival and induces specific tolerance (Akalin et al., 1996; Lenschow et al., 1995; Sayegh, 1998). Therefore, the genes of the costimulatory molecules were candidate for a large spectrum of autoimmune diseases such as rheumatoid arthritis (Matsuchita et al., 2000), asthma (Cotydon et al., 2007), systemic lupus erythematosus (Wong et al., 2005) or multiple sclerosis (Van Veen et al., 2003). Thus, numerous polymorphic markers have been reported to cause abnormal expression or dysfunction of the proteins.

3. Polymorphisms in the CTLA-4, CD28, and CD86 genes

The CTLA-4 and the CD28 genes are homologous and closely linked in the 2q33 chromosomal region. CD28 is constitutively expressed on almost all resting human CD4+ T-cells and 50–80% of all CD8+ T-cells. In contrast, CTLA-4 is only expressed after T-cell activation. It is usually located intra-cellularly and moves rapidly to the cell surface at the site of T-cell/APC interaction (Bier, 2007). CD80 and CD86 are encoded by homologous genes located in the 3q21 chromosomal region (Dalla-Costa et al., 2010). The expression kinetics of CD80 and CD86 also differ: CD86 is constitutively expressed at low levels and rapidly up-regulated, whereas CD80 is inducibly expressed later than CD86 on APCs (Greenwald et al., 2002).

Among the most characterized polymorphisms in CTLA-4 costimulatory molecule, an A/G transition at position (+49) of the exon 1 (rs231775) causes a Threonine/Alanine substitution in the leader peptide and could affect the inhibitory function of CTLA-4. The (+49) A allele had been identified as protective, whereas the G allele was associated with greater susceptibility to autoimmune diseases (Liu et al., 2010). A single nucleotide polymorphism (SNP) at position (-318) C/T in the promoter region (rs5742909) influences promoter activity and the expressions of both CTLA-4 mRNA in un-stimulated cells and cell-surface CTLA-4 on activated cells (Kusztal et al., 2010). The (+6230) A/G polymorphism (or CT60) in the 3'untraslated region (UTR) of the CTLA-4 gene (rs3087243) has been shown to be associated with the mRNA level of soluble CTLA-4. This later SNP was associated with the levels of membrane and cytoplasmic CTLA-4 in CD4 T lymphocytes from multiple sclerosis patients and with the variation of serum soluble CTLA-4 level in Graves' disease patients (Kusztal et al., 2010). Also in the 3'UTR of CTLA-4 gene, a microsatellite (AT)n has been identified at position 642. The variation in the number of the dinucleotide (AT) repeat is associated with the stability of mRNA transcripts (Liu, 2010). It has been established that a low number of (AT) repeats is responsible for favorable mRNA stability, which results in lower T cell proliferation (Kusztal et al., 2010).

For the CD28 gene, a T/C SNP at position (+17) of the intron 3 (rs3116496) or IVS3 (+17) is identified. The functional role of this SNP is not yet clearly established, but it is known that this polymorphic site is within a region where regulatory elements could bind (Marín et al., 2005).

In the CD86 gene, a (+1057) G/A polymorphism in the exon 8 (rs1129055) results in an Alanine/Threonine substitution at codon 304 located in the CD86 cytoplasmic tail which contains putative phosphorylation sites for protein kinase C (PKC). This substitution could modify the phosphorylation level in this region and influence the APC-signal transmission pathway by CD86 (Pawlak et al., 2010).

Since the regulation of T lymphocytes activation is crucial during the allograft rejection process, we focused on the relationship between CTLA-4, CD28 and CD86 gene polymorphisms and renal transplant outcomes. Herein, we examined the genotypic

distribution of the (+49) A/G, (-318) C/T, (+6230) G/A and (AT)n microsatellite of CTLA-4, (+17) T/C of CD28 and (+1057) G/A of CD86 gene in a cohort of Tunisian kidney allograft recipients.

4. Subjects and methods

4.1 Patients

We retrospectively investigated 127 renal transplant recipients who underwent transplantation between 1986 and 2008. Based on the important impact of HLA disparity between donor and recipient on transplantation outcomes, patients were classified into two groups according to the HLA-haplotype similarity between donor and recipient:

- Group I: included 23 HLA-identical haplotype allograft recipients
- Group II: included 104 recipients showing one or more mismatches in the HLA haplotype.

The acute rejection diagnosis was made by clinical, histological, and biochemical standard assessment (Banff criteria). As maintenance immunosuppression, all patients received prednisolone, tacrolimus and/or mycophenolate mofetil. Patients of Group II received rabbit anti-thymocyte globulin (ATG) as induction therapy. ATG was also administrated after an acute rejection episode in 13 patients of Group I and 8 of Group II.

Prior to transplantation, the sera of 124 patients, 23 from Group I and 101 from Group II, were tested for anti-HLA antibodies by the microlymphocytotoxicity assay. The main characteristics of our patients are summarized in Table 1.

Features	*Patients (n=127)*	*Group I (n=23)*	*Group II (n=104)*
Age (years)	32.08±10.92	31.74± 6.70	32.36±11.51
Sex ratio (Males/ Females)	1.71	2.22	1.60
Donor type (%)			
RLD	79.53	100	75
DD	16.53	0	20.19
ULD	3.94	0	4.81
Fallow-up time (months)	85 ± 63.59	129±73.83	75±57.06
Initial nephropathy (%)			
Glomerulonephritis	40.94	56.52	37.35
Unspecified Chronic nephropathy	28.35	4.35	33.65
Tubulointerstitial nephropathy	18.11	13.04	16.35
Vascular nephropathy	7.09	8.07	6.73
Rapidly evolutionary nephropathy	3.15	-	2.89
Gravidic nephropathy	0.79	-	0.96
Hereditary nephropathy	0.79	-	0.96
Hemolytic and uremic syndrome	0.79	-	0.96
Acute rejection (%)	31 (24.41)	5 (21.74)	26 (25)
Anti-HLA antibodies presence in pre-transplantation	19/124	4/23	15/101

Table 1. Epidemiological features of the kidney allograft recipient
RLD: related living donor; DD: deceased donor; ULD: unrelated living donor.

4.2 Controls

As a control group, we examined 83 ethnically and geographically matched healthy subjects recruited from the blood donors of the same area than patients. The study was approved by the local ethics committee

4.3 Molecular biology methods

4.3.1 DNA extraction

Genomic DNA was isolated from EDTA-anticoagulated peripheral blood samples of unrelated healthy blood donors and renal recipients, and extracted by a standard salting-out procedure.

4.3.2 Polymorphisms genotyping

4.3.2.1 CTLA-4 (+49) A/G and CTLA-4 (+6230) A/G

Typing of the CTLA-4 exon 1 A/G transition at position 49 and 3'UTR (+6230) A/G polymorphism was achieved by the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method using a PCR System 2700 Thermal Cycler (Applied Biosystems, Gene Amp®). The PCR protocol and the primers used are listed in Table 2.
PCR was carried out in a final volume of 20 µl containing 100ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 10 pmol of each primer and 0.5U of Taq DNA polymerase (Promega, USA). The restriction enzymes used were *Kpn I* for (+49) A/G and *Nco I* for (+6230) A/G.

4.3.2.2 CTLA-4 (-318) C/T and CD28 (+17)T/C

To determine genotypes of the CTLA-4 (-318) C/T in the gene promoter of CTLA-4 and of the CD28 (+17) T/C polymorphism a PCR-SSP (sequence specific primer) was used in a 25µl final volume mixed solution, containing 100 ng genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 10 pmol of each the various allele-specific forward and reverse primers, and 0.5U of Taq DNA polymerase (Promega, USA). The PCR protocol and the primers used are listed in Table 2.

4.3.2.3 CTLA-4 (AT)n

The genotyping of (AT) repeat in the UTR of the CTLA-4 gene was performed by length-fragments analysis method using an automated sequencer (Perkin-Elmer ABI Prims 310 Genetic Analyzer) and Peak Scanner software. A lane size standard (ROX-500) and the Formamide Hi-Di were added to all samples according to each manufacturer's instructions. A PCR was previously realized in a final volume of 20 µl containing 50 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 10 pmol of each primer and 0.5U of Taq DNA polymerase (Promega, USA) with the protocol and the couple of primers summarized in Table 2.

4.3.2.4 CD86 (+1057) G/A

For the amplification of a DNA fragment containing the (+1057) G/A polymorphism on the exon 8 of the CD86 gene, a PCR was performed using a PCR System 2700 Thermal Cycler (Applied Biosystems, Gene Amp®) in a final volume of 20µl containing 50ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 10 pmol of each primer and 0.5U of Taq DNA polymerase (Promega, USA) with the protocol and the couple of primers summarized in Table 2. The amplified fragments were then sequenced in forward direction using the Ex 8/3 primer in an ABI PRISM Dye Terminator Cycle Ready Reaction kit (Applied Biosystems) under recommended conditions. Sequenced samples were purified using Centri-Sep columns (Dye EXTm 2.0 Spin Kit, Qiagen) according to manufacturer's instructions, loaded in a PE ABI Prisms 310 Genetic Analyzer (Perkin Elmer) and analyzed

using ABI Prisms Navigator Software. The two alleles G and A at position (+1057) were observed as different fluorescence peaks in that position.

4.3.3 Statistical analysis

Allelic and genotypic frequencies were evaluated by direct counting. Statistical comparisons were performed, between patients and controls, by χ^2 test calculated on 2x2 contingency tables using the Statcalc program (Epi Info version 20010; Centers for Disease Control and Prevention, Atlanta, GA, USA). Fisher's exact test was used when an expected cell value was less than 5. p value < 0.05 was considered to be statistically significant. Odd ratio(OR), with 95% confidence intervals (95%CI), was calculated using the same software.
Haplotype frequencies and Hardy-Weinberg equilibrium *p* values were estimated by the Thesias 3.1 program (DA Tregouet, INSERM U525, Paris, France).

Polymorphisms	*Primers*	*Temperature, time and cycles for PCR*
CTLA-4 (+49) A/G	**Forward:** 5′CAAGGCTCAGCTGAACCTGGGT3′ **Reverse:** 5′TACCTTTAACTTCTGGCTTTG3′	Initial denaturation for 4 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 67°C and 1 min at 72°C and final elongation 5 min at 72°C.
CTLA-4 (-318) C/T	**Specific primers:** F10: 5′ ACTTAGTTATCCAGATCCAC 3′ F11: 5′ ACTTAGTTATCCAGATCCAT 3′ **Common primer:** R10: 5′ AGGCTCTTGAATAGAAAGC 3′	Initial denaturation for 2 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C and 45 sec at 72°C and final elongation 2 min at 72°C.
CTLA-4 (+6230) A/G	**Forward** CT60 Sens: 5′ CACCACTATTTGGGATATACC 3′ **Reverse** CT60 Antis: 5′ AGCTCTATATTTCAGGAAGGC 3′	Initial denaturation for 5 min at 94°C, 30 cycles of 40 sec at 94°C, 30 sec at 61°C and 50 sec at 72°C and final elongation 7 min at 72°C.
CTLA-4 (AT)n	**Forward** (AT)n R: 5′TGGTGTATTAGTGTCCTG 3′ **Reverse** (AT)n F: 5′ Fam GATGCTAAAGGTTGTATT 3′	Initial denaturation for 2 min at 94°C, 3 cycles of 30 sec at 94°C, 30 sec at 54°C and 30 sec at 72°C and final elongation 7min at 72°C.
CD28 (+17) T/C	**Specific primers:** CD28 T: 5′ CTGGGTAAGAGAAGCGCAAT 3′ CD28 C: 5′ CTGGGTAAGAGAAGCGCAAC 3′ **Common primer:** CD28 Com: 5′ CTCAATGCCTTCTGGAAATC 3′	Initial denaturation for 2 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C and final elongation 5 min at 72°C.
CD86 (+1057) G/A	**Forward** Ex 8/3 5′CTCCTCATTGCTGTTCCAATGGCAACC 3′ **Reverse** Ex 8/4 5′CATGAGCCATTAAGCTGGGCTTGGCCC 3′.	Initial denaturation for 4 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 67°C and 1 min at 72°C and final elongation 5min at 72°C.

Table 2. Sequences of primers and PCR conditions

5. Results

5.1 Patient characteristics

No significant differences were found between groups of patients in gender, age, ethnicity or primary disease (Table 1).

Thirty-one patients (24.41%) developed at least one acute rejection episode within the first six months after transplantation. The rate of acute rejection was lower in Group I (21.74%) than in Group II (25%) or in the global group of patients (24.41%) (Table 1).

Before transplantation, 19/124 recipients were sensitized (anti-HLA positive): 4/23 in Group I, two of them developed an acute rejection episode and 5/101 in Group II, including 7 with an acute rejection episode. Among the 105 anti-HLA negative recipients, 19 were in Group I (3 of them undergone an acute rejection) and 86 were in Group II (18 with acute rejection).

The incidence of acute rejection was statistically associated with the presence of anti-HLA antibodies before transplantation in the 124 patients (p=0.01) and in Group II (p=0.04) (Table3).

	Patients (n=124)		*Group I (n=23)*		*Group II (n=101)*	
	AR (+) n= 30	**AR (-) n= 94**	**AR(+) n=5**	**AR (-) n= 18**	**AR(+) n= 25**	**AR (-) n= 76**
AHA(+)	9	10	2	2	7	8
AHA (-)	21	84	3	16	18	68
p	0.01[a]		0.19		0.04[b]	

AHA: anti-HLA antibodies; AR(+): acute rejection; AR(-): non acute rejection

[a] : p = 0.01; OR=3.60; 1.12<OR<11.20; 95% CI

[b] : p = 0.04; OR=3.31; 0.88<OR<11.91; 95% CI

Table 3. Association between acute rejection and the presence of anti-HLA antibodies in pre-transplantation

5.2 Distribution of genotypes and alleles frequencies in patients and controls

The genotyping of the microsatellite (AT)n in the UTR of the CTLA-4 gene showed 22 distinct alleles of size ranging from 88 to 146 bp. The shortest allele of 88 bp was the most frequent in controls and patients.

For all polymorphisms, no differences were found in the frequencies of genotypes and alleles between the global cohort of patients and controls (Table 4).

5.3 Polymorphisms and acute rejection

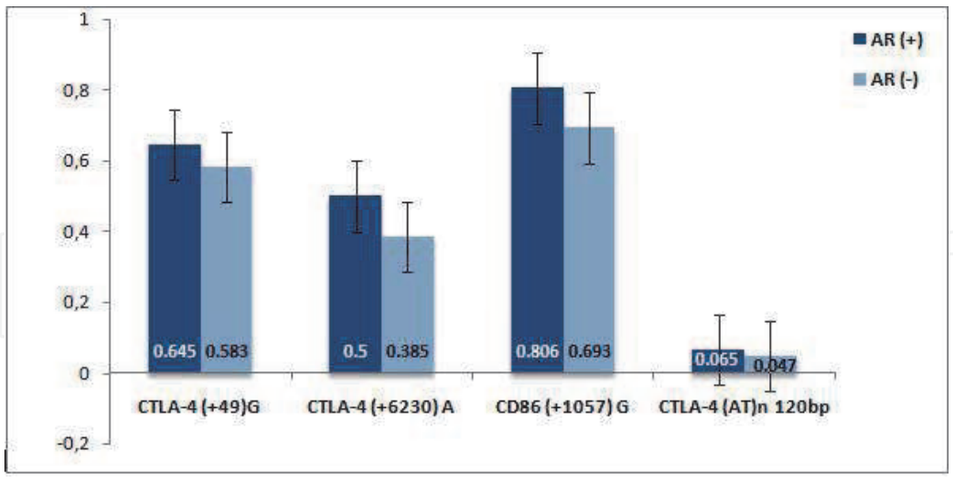
Comparing the genotypes distribution between acute rejection and non acute rejection patients, we noticed that the (+49) G/G and (+6230) A/A genotypes of the CTLA-4 gene and the (+1057) G/G genotype on the CD86 gene were more frequent in case of acute rejection (Table 5).

The frequencies of the alleles: (+49) G, (+6230) A and (AT) 120bp of the CTLA-4 gene and of (+1057) G allele of the CD86 gene were higher in acute rejection patients than in non-acute rejection ones (Figure 3).

Polymorphisms	*Patients (n=127)*	*Controls (n=83)*	*p (HWE)*
CTLA-4 (+49)A/G			
Genotypes			
A/A	18.90	7.23	
A/G	42.52	37.35	
G/G	38.58	55.42	
Alleles			
A	0.402	0.260	0.194
G	0.598	0.740	
CTLA-4 (-318) C/T			
Genotypes			
C/C	88.98	90.36	
C/T	10.24	8.43	
T/T	0.78	1.20	
Alleles			
C	0.941	0.946	0.373
T	0.059	0.054	
CTLA-4 (+6230) A/G			
Genotypes		2	
G/G	34.65	5.03	
A/G	48.03	48.19	
A/A	17.32	26.50	
Alleles			
G	0.587	0.493	0.913
A	0.413	0.506	
CTLA-4 (AT)n			
Genotype			
88 bp/88 bp	17.32	29.07	
Allele	0.417	0.518	
88 bp			
CD28 (+17) T/C			
Genotypes			
T/T	48.03	55.42	
T/C	33.07	38.55	
C/C	18.90	6.02	
Alleles			
T	0.646	0.747	0.001
C	0.354	0.253	
CD86 (+1057) G/A			
Genotypes	52.76	60.24	
G/G	38.58	36.14	
G/A	8.66	3.62	
A/A			
Alleles			
G	0.720	0.783	0.635
A	0.280	0.217	

p (HWE): Hardy-Weinberg equilibrium *p* value

Table 4. Genotypes and alleles frequencies in patients and controls (%)



AR(+): acute rejection; AR(-): non acute rejection

Fig. 3. Allele frequencies in acute rejection and in non-acute rejection patients

On the other hand, no differences were found in the distribution of alleles or genotypes frequencies of (-318) C/T polymorphism of the CTLA-4 gene or (+17) T/C of the CD28 gene. In addition, in negative anti-HLA recipients (105 patients), the 120 bp allele of (AT)n microsatellite in CTLA-4 gene was more frequent in acute rejection patients (0.071) than in non-acute rejection ones (0.055).

Moreover, in the negative anti-HLA recipients of the Group I (19 patients), the alleles (+49) G and (+6230) A of CTLA-4 were also more frequent in acute rejection patients than in non-acute rejection ones (0.833 *vs* 0.625 and 0.5 *vs* 0.281 respectively).

Polymorphisms	*Patients (n = 127)*		*Group I (n = 23)*		*Group II (n =104)*	
	AR(+) n=31 n(%)	*AR(-) n=96 n(%)*	*AR(+) n=5 n(%)*	*AR(-) n=18 n(%)*	*AR(+) n=26 n(%)*	*AR(-) n=78 n(%)*
CTLA-4 (+49) A/G						
Genotypes						
G/G	14 (46.16)	35 (36.46)	4 (80)	6 (33.33)	10 (38.46)	29 (37.18)
G/A	12 (38.71)	42 (43.75)	1 (20)	11 (61.11)	11 (42.31)	31 (39.74)
A/A	5 (16.13)	19 (19.79)	0	1 (5.56)	5 (19.23)	18 (23.08)
Alleles						
G	0.645	0.583	0.900	0.689	0.596	0.570
A	0.355	0.417	0.100	0.361	0.404	0.430
CTLA-4 (-318) C/T						
Genotypes						
C/C	27 (87.1)	86 (89.58)	4 (80)	16 (88.89)	23 (88.46)	70 (89.74)
C/T	4 (12.9)	9 (3.38)	1 (20)	1 (5.56)	3 (11.54)	8 (10.26)
T/T	0	1 (1.04)	0	1 (5.56)	0	0
Alleles						
C	0.935	0.943	0.900	0.917	0.942	0.949
T	0.065	0.057	0.100	0.083	0.058	0.051

CTLA-4 (+6230) G/A						
Genotypes						
G/G	8 (25.81)	36 (37.50)	0	8 (44.44)	8 (30.77)	28 (35.90)
G/A	15 (48.38)	46 (47.92)	4 (80)	10 (55.56)	11 (42.31)	36 (46.15)
A/A	8 (25.81)	14 (14.58)	1 (20)	0	7 (29.92)	14 (17.95)
Alleles						
G	0.500	0.615	0.400	0.722	0.519	0.590
A	0.500	0.385	0.600	0.278	0.481	0.410
CTLA-4 (AT)n						
Genotypes						
88bp/88bp	7 (22.58)	15 (15.62)	1 (20)	1 (5.56)	6 (23.08)	14 (17.95)
120bp/120bp	0	1 (1.04)	0	0	0	1 (1.18)
120bp/other	7 (22.58)	21 (21.88)	1 (20)	5 (27.78)	5 (19.23)	6 (7.69)
Allele 120 bp	0.065	0.047	0	0.138	0.115	0.155
CD28 (+17) T/C						
Genotypes						
T/T	14 (46.16)	47 (48.96)	1 (20)	9 (50)	13 (50)	38 (48.27)
T/C	12 (38.71)	30 (31.25)	3 (60)	2 (11.11)	9 (34.62)	28 (35.90)
C/C	5 (16.13)	19 (19.79)	1 (20)	7 (38.89)	4 (15.38)	12 (15.38)
Alleles						
T	0.645	0.646	0.500	0.556	0.673	0.667
C	0.355	O.354	0.500	0.444	0.327	0.333
CD86 (+1057) A/G						
Genotypes						
G/G	20 (64.52)	47 (48.96)	3 (60)	8 (44.44)	17 (65.38)	39 (50)
G/A	10 (32.26)	39 (40.62)	1 (20)	7 (38.89)	9 (34.62)	32 (41.03)
A/A	1 (3.22)	10 (10.42)	1 (20)	3 (16.17)	0	7 (8.97)
Alleles						
G	0.806	0.693	0.700	0.639	0.827	0.705
A	0.914	0.307	0.300	0.361	0.173	0.295

Table 5. Genotypes frequencies in renal transplant recipients: comparison between acute rejection (AR+) and non acute rejection (AR-)

Haplotype	*Patients*		*Group I / anti-HLA(-)*	
	RA(+) n=31	*RA(-) n=96*	*RA(+) n=3*	*RA(-) n=16*
AGA	0.041	0.084	0.167	0.177
AGG	0.235	0.266	-	0.198
AAA	0.023	0.027	-	-
AAG	0.055	0.040	-	-
GGA	0.098	0.129	0.042	0.063
GGG	0.125	0.136	0.291	0.280
GAG	0.390	0.251	0.209	0.177
GAA	0.031	0.068	0.291	0.103

Table 6. Haplotype frequencies of the SNPs (+49) A/G and (+6230) G/A of CTLA-4 and (+1057) A/G of CD86 in acute rejection and non acute rejection patients

We also estimated the haplotype frequencies considering the three SNPs: (+49) A/G and (+6230) G/A of CTLA-4 and of CD86 in acute rejection and non acute rejection recipients (Table6). Our results showed that the association CTLA-4(+49)G/CTLA-4(+6230)A/ CD86(+1057)G was more frequent in patients who undergone an acute rejection comparing with those who do not in the total cohort: 0.390 *versus* 0.251, and especially in the anti-HLA negative patients of the Group I: 0.209 *versus* 0.177.

6. Discussion

The interactions of CTLA-4 and CD28 with their ligands CD80 and CD86 represent an important co-stimulatory signal that regulates T-cell activation. The genes encoding these molecules may be candidates for the susceptibility to acute renal allograft rejection. In our study, we sought to investigate the most characterized polymorphisms in the CTLA-4, the CD28 and the CD86 genes among 83 healthy subjects and 127 renal transplants.

Based upon the important impact of HLA disparity between donor and recipient on transplantation outcomes, we subdivided the patients into two groups. Indeed, the rate of acute rejection in our cohort was lower in the recipients showing similar HLA haplotype with their donors.

It is also established that pre-formed anti-HLA antibodies in allograft recipients can induce sever vascular disease of organ transplant, particularly an antibody-mediated rejection (humoral rejection), which has become a major clinical challenge since the rejection caused by antibodies resists treatment by conventional drug regimens. In renal transplant, hyper acute rejection is the most often caused by anti-HLA antibodies produced by the graft recipient (Chang et al., 2009; Poli, 2009). Moreover, donor-specific anti-HLA antibodies developed after transplantation transduce signals that are both pro-inflammatory than pro-proliferative suggesting mechanistic roles in acute and chronic antibody-mediated rejection (Li et al., 2009). Thus, antibody response to donor HLA class I and class II antigens represents a significant risk factor for kidney transplant failure. That's why, prior to transplantation, recipient sera are tested for HLA antibodies and a determination of donor mismatch acceptability increases transplant success. Sensitization occurs through exposure to HLA antigens via blood transfusions, previous grafts, or pregnancies (Karahan et al., 2009). In our study, sensitized patients were statistically more susceptible to develop acute rejection than non-sensitized ones. This result corroborates those of previous studies which reported that patients with pre-transplantation high levels of panel-reactive antibody show an increased risk of graft failure (Chang et al., 2009; Karahan et al., 2009).

A recent analysis in end-stage renal disease patients among Turkish population proposed also that de novo synthesis of these antibodies after transplantation could be detrimental for the graft. However, we did not detect a significant difference in acute rejection incidence between patients which developed anti-HLA antibodies in post transplantation and those which do not. In fact, the influence of anti-HLA antibodies on the development of rejection episodes depends probably on patient-specific clinical factors differing among patients (Karahan et al., 2009).

The distribution of genotype and allele frequencies in our study highlighted a trend toward an increase of CTLA-4 (+49) G, (+6230) A, 120 bp (AT)n and in CD86 (+1057) G alleles frequencies among patients with acute allograft loss compared to non-acute rejection ones, suggesting that these alleles may enhance the incidence of acute rejection. In order to validate this supposition, we sought to eliminate the other risk factors: the presence of anti-

HLA antibodies and the HLA disparity between donor and recipient. Thus, we compared allele frequencies in non-sensitized patients of the Group I, and we also noticed the rise of these same alleles with the occurrence of acute rejection. However, the wide confidence intervals (CIs) in this analysis reflect the relatively small numbers in some of the subgroups, with a consequent loss of statistical power. A larger patient cohort would therefore be required for us to reduce the width of the CIs and confirm these observations.

Our findings can join those of Kim et al. who reported the implication of the (+49) G allele of CTLA-4 in late acute rejection kidney allograft (Kim et al., 2010), and also those of Marìn et al. who revealed an association of the (+1057) G allele of CD86 and acute rejection in liver allograft (Marìn et al., 2005). In contrast to us, Muro et al. study shows that the potential correlated allele of CTLA-4 gene to liver acute rejection is the (+6230) G (Muro et al, 2008). Slavcheva et al. rather revealed a lack of association between CTLA-4 (+49) A/G polymorphism and renal or liver acute rejection, and a possible implication of alleles 92 and 94 bp of the (AT)n microsatellite (Slacheva et al., 2001).

Our study did not reveal any differences between patients with an acute rejection episode and those free of acute rejection in genotypic or allelic frequencies of the (-318) C/T gene promoter of the CTLA-4 or the (+17) T/C in the intron 3 of the CDE28 gene. Whereas, a recent study reported that the (-318) T allele induces a more important activity of the promoter region, a higher level of CTLA-4 molecular expression, and a more efficient inhibitory effect on T lymphocyte activation than the (-318) C allele (Slacheva et al., 2001). Besides, this SNP has been associated to several autoimmune disorders such as chronic obstructive pulmonary disease (Liu et al., 2010) or sporadic breast cancer (Fan et al., 2004). The exact role of the (+17) T/C polymorphism the CD28 gene is not really clear. It has been rarely correlated directly to autoimmunity dysfunction. Recently, a Polish study on cervical squamous cell carcinoma (CSCC) reported a potential association with the CD28 (+17) T/C polymorphism, but the authors specified that this association is restricted only to the well-differentiated CSCC (Pawlak et al. 2010).

The analysis of the haplotype frequencies distribution in our recipients revealed that the combination of CTLA-4(+49) G/CTLA-4 (+6230) A/CD86 (+1057) G was more frequent in case of acute rejection considering the whole cohort or the anti-HLA (-) patients of the Group I. These results led us to suppose that the simultaneous presence of the alleles CTLA-4 (+49) G, (+6230) A, (AT) 120bp and CD86 (+1057) G could constitute susceptibility haplotype to acute rejection in renal allograft. In fact, alleles at different loci may interact, increasing or decreasing susceptibility to a disease [22]. Thus, we know that the SNPs (AT)n and (+6230) A/G are involved in both the level and the stability of mRNA of the CTLA-4 molecule. Therefore, we suggest that the association CTLA-4 (+49) G-(+6230) A-(AT) 120bp may be linked to reduced expression of CTLA-4 on the T-cell surface, which could lead to reduce CTLA-4 production and subsequently impair inhibitory function and contribute to enhance T lymphocytes proliferation. The phosphorylation site introduced in the cytoplasmic tail of the CD86 molecule caused by the (+1057) A/G SNP affects APC signal transduction. So it is likely to presume that (+1057) G allele leads to a higher capability of T cells activation. Consequently, patients bearing concurrently the CTLA-4 (+49) G, (+6230) A, (AT) 120bp and CD86 (+1057) G alleles could develop elevated level of immune response and then a higher risk of allograft rejection. However, these results should be interpreted with caution due to the low number of patients in some subgroups. This hypothesis can confirm a recent research realized in the same context which investigated the influence of

CTLA-4 gene polymorphism on long-term kidney allograft function. No significant correlation of a particular SNP with the risk of acute rejection was found. However, the combination (+49)AA/(AT)LL haplotype (where L=low AT repeat number) was associated to better allograft function than the GG/HH haplotype (where H=high ,i.e. >82-bp repeats) (Kusztal et al., 2010).

7. Conclusion

In summary, our analysis of the costimulatory molecules genes revealed a genetic profile which may be implied in the enhancement of acute rejection incidence in Tunisian kidney recipients. We suggest that the alleles (+49) G, (+6230) A and (AT) 120bp of CTLA-4 gene and (+1057) G of CD86 gene may have a predisposition effect on acute rejection acting independently of the other risk factors: the presence of anti-HLA antibodies and the HLA disparity between donor and recipient. The (-318) C/T SNP in the promoter of CTLA-4 gene and the (+17) T/C in the CD28 gene seem to not be involved in the susceptibility to renal allograft rejection. More studies are needed in order to clarify if determination of the costimulatory molecules genotypes could be a useful prognostic factor witch interfere in allograft failure.

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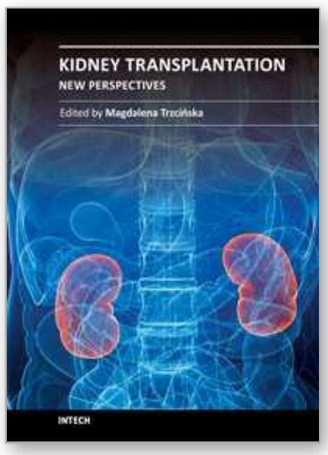
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Although many years have passed since the first successful kidney transplantation, the method, although no longer considered a medical experiment, is still perceived as controversial and, as such, it triggers many emotions and thatâ€™s why conscious educational efforts are still needed for kidney transplantation, for many people being the only chance for an active lifestyle and improved quality of life, to win common social acceptance and stop triggering negative connotations. Apart from transplantation controversies piling up over years transplantologists also have to face many other medical difficulties. The chapters selected for this book are of high level of content, and the fact that their authors come from many different countries, and sometimes even cultures, has facilitated a comprehensive and interesting approach to the problem of kidney transplantation. The authors cover a wide spectrum of transplant-related topics.

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