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LIVER DISEASE

Polymorphisms in the T cell regulatory gene cytotoxic T lymphocyte antigen 4 influence the rate of acute rejection after liver transplantation

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Background: The cytotoxic T lymphocyte antigen 4 (CTLA-4) gene encodes for a membrane bound (mCTLA-4) and a soluble (sCTLA-4) isoform, which are both involved in regulation of T cell function. The CTLA-4 +49A/G single nucleotide polymorphism (SNP) influences expression of mCTLA-4; +6230G/A SNP affects the production of sCTLA-4.

Aim: To examine whether these functional SNPs influence the rate of rejection after liver transplantation.

Patients and methods: Liver graft recipients (n = 483) were genotyped for both SNPs, and haplotypes were reconstructed. Association with rejection was tested by the log rank test using the Kaplan-Meier method with time to the first acute rejection episode as outcome. Multiple analysis of SNPs together with demographic factors was performed by Cox regression.

Results: Three haplotypes were observed in the cohort: +49A/+6230A, +49A/+6230G, and +49G/+6230G. The +49A/+6230G haplotype was significantly and dose dependently associated with acute rejection (p = 0.01). Of the demographic factors tested, only underlying liver disease was significantly associated with rejection. Adjusted for underlying liver disease, each additional +49A/+6230G haplotype allele resulted in a significantly higher risk of acute rejection (risk ratio 1.34 (95% confidence interval 1.04–1.72); p = 0.02). Patients who lacked this haplotype had the lowest, carriers an intermediate, and homozygotes the highest risk of acute rejection.

Conclusion: The CTLA-4 +49A/+6230G haplotype, which encodes for normal mCTLA-4 expression but reduced sCTLA-4 production, is a co-dominant risk allele for acute rejection after clinical liver transplantation. This implies that even under immunosuppression, CTLA-4 is critically involved in the regulation of the human immune response to allogeneic grafts.

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Cytotoxic T lymphocyte antigen 4 (CTLA-4; CD152) is a homologue of CD28, which is expressed on the cell surface of activated memory T cells and on $CD4^+CD25^+$ regulatory T cells, and is critically involved in downregulation of T cell responses. Several mechanisms may account for its inhibitory effects. Firstly, CTLA-4 has a higher affinity for the B7 molecules CD80 and CD86 compared with CD28, and thereby serves as a competitive antagonist of CD28 for B7 binding.[1] Secondly, on binding to B7 molecules, CTLA-4 actively suppresses interleukin (IL)-2 production and cell cycle progression of T cells.[2] Thirdly, CTLA-4 is one of the inhibitory molecules by which $CD4^+CD25^+$ regulatory T cells exert their suppressive function on effector T cell activation.[3] Finally, an alternative splice form of CTLA-4, which is secreted by resting T cells,[4] can suppress allogeneic T cell activation.[5] This soluble CTLA-4 (sCTLA-4) isoform is present in human serum[4] and its levels are enhanced in the serum of patients with autoimmune thyroid disease.[6]

Several autoimmune diseases have been found to be associated with allelic variations in the CTLA-4 gene. The strongest associations have been observed with the single nucleotide polymorphisms (SNPs) CTLA-4 +49A/G and +6230G/A. The +49 A/G SNP results in substitution of threonine by alanine in the leader peptide of the newly formed CTLA-4 molecule. It was found to be associated with, for example, Graves' disease,[7] diabetes mellitus type 1,[8] primary biliary cirrhosis,[9] and autoimmune hepatitis.[10] The +6230G/A SNP is situated in the 3' untranslated region of the CTLA-4 gene, and was recently found to be more strongly associated with Graves' disease compared with the +49A/G SNP.[11] In addition, associations between the +6230G/A SNP and type 1 diabetes,[12] and clearance of hepatitis B virus (HBV) infection[13] were found.

In view of the important role of CTLA-4 in regulating rejection activity against allogeneic organ grafts in experimental animals,[14][15] we examined whether genetic variations in the CTLA-4 gene influenced the rate of acute rejection after liver transplantation. In a previous single centre study aiming to explore whether SNPs in costimulatory molecules influenced the risk of acute rejection after liver transplantation, we found evidence for an association of the +49A/G SNP with rejection.[16] Here we present data from a multicentre study with a larger cohort of patients which aimed to determine to what extent the functional CTLA-4 +49A/G and +6230G/A SNPs influence the probability of rejection after liver transplantation.

PATIENTS AND METHODS
Patients
In this retrospective study, 483 liver transplant recipients, derived from three centres (126 from Rotterdam, 204 from Birmingham, and 153 from Newcastle), who received an orthotopic liver transplant between 1987 and 2001, were

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Abbreviations: CTLA-4, cytotoxic T lymphocyte antigen 4; mCTLA-4, membrane CTLA-4; sCTLA-4, soluble CLTA-4; SNP, single nucleotide polymorphism; HGH, human growth hormone; IL, interleukin; HBV, hepatitis B virus; HCV, hepatitis C virus; PCR, polymerase chain reaction

Table 1 Polymerase chain reaction (PCR) primers used in the study

SNP	PCR primer sequences	Annealing temp (℃)
	Primers used for allele specific PCR	
CTLA-4 +49A/G	5′ CAAGCCAGATTGGAGTTTA 3′	T1 = 64
	5′ CTCAGCTGAACCTGGCTA/G 3′	T2 = 58
CTLA-4 +6230G/A	5′ AGGAAGGCAGATCAAAATGC 3′	T1 = 65
	5′ CACCACTATTTGGGATATAACA/G 3′	T2 = 59
HGH	5′ GCCTTCCCAACCATTCCCTTA 3′	
	5′ TCACGGATTTCTGTTGTGTTTC 3′	
	Primers used for PCR-RFLP	
CTLA-4 +49A/G	5′ CCACGGCTTCCTTTCTCGTA 3′	56
	5′ AGTCTCACTCACCTTTGCAG 3′	
CTLA-4 +6230G/A	5′ CATCCTTGCATTTGAATATTGTG 3′	59
	5′ AAGAATCACAGAGGGCCCAGTG 3′	

CTLA-4, cytotoxic T lymphocyte antigen 4; SNP, single nucleotide polymorphism; HGH, human growth hormone; RFLP, restriction fragment length polymorphism.

included. Patients with a follow up period of less than 90 days and patients treated with anti-IL-2 receptor monoclonal antibodies as part of their immunosuppressive induction treatment were excluded from the study. All included patients received standard immunosuppressive therapy consisting of ciclosporin or tacrolimus and prednisone, with or without azathioprine. Mean follow up was five years. Written consent was obtained from all participating patients, and the Medical Ethics Committee of the Erasmus MC in Rotterdam approved the study. Patient characteristics and data on acute rejection were derived from computerised databases or extracted from patient files. To correct for a possible influence of underlying liver disease on the risk of rejection, patients were classified according to their diagnosis for liver transplantation into the following categories: viral liver infection (acute or chronic HBV or hepatitis C virus (HCV) infection), autoimmune related liver diseases (primary biliary cirrhosis, primary sclerosing cholangitis, or autoimmune hepatitis), alcoholic liver cirrhosis, fulminant hepatic failure, or other (which contained all other diagnoses). Acute rejection was defined as an episode with increasing liver enzymes with no other identifiable cause, together with moderate or severe rejection activity in a liver biopsy, which was responsive to high dose corticosteroid or antilymphocyte immunoglobulin treatment.

Determination of SNPs

DNA was isolated from blood by the classic salting out method, involving proteinase K digestion and ethanol precipitation. Samples from Rotterdam and Birmingham were analysed in Manchester by allele specific polymerase chain reaction (PCR) by one of the investigators (ÖT) who was blinded to the clinical data. Allele specific forward primers and generic reverse primers were designed using the sequence of the CTLA-4 gene published in the Nucleotide Data bank (http://www.ncbi.nlm.nih.gov). The sequences of the primers are depicted in table 1. Each of the allele specific primers was mixed with the associated generic primer in a 1:1 ratio and separate reactions were performed for determination of each allele. An internal control amplification of the human growth hormone (HGH) gene was used as a positive control for the PCR reaction. DNA was amplified in Mastermix (Abgene, Epsom, UK) supplemented with 5 μM CTLA-4 and HGH primers. PCR was run in a PTC-100 PCR machine (MJ Research, Massachusetts, USA) according to the following programme: 95℃ 60 seconds, 10 cycles of 95℃ 15 seconds, T1℃ 50 seconds, 72℃ 40 seconds, and thereafter 20 cycles of 95℃ 20 seconds, T2℃ 50 seconds, and 72℃ 50 seconds. The primer specific annealing temperatures T1 and T2 are depicted in table 1. PCR products were monitored

after electrophoresis on a 2% agarose gel containing ethidium bromide.

Samples from Newcastle were genotyped by a PCR-restriction fragment length polymorphism method.[10] The sequences of the primers used to amplify the SNP flanking regions are shown in table 1. *Bbv*I was used to digest the G allele of +49 SNP and *Hpy*CH4IV to digest the G allele of the +6230 SNP. Alleles were identified after electrophoresis in an agarose gel.

Haplotype-genotype estimation

The linkage disequilibrium coefficient (D′) between the two SNPs was calculated using Phase software version 2[17] (http://archimedes.well.ox.ac.uk/pise/PHASE.html). This program was also used to estimate the most possible haplotype pair for each individual.

Statistical analysis

The Kaplan-Meier method was used to determine the cumulative probability of the first episode of acute rejection in patient groups with different genotypes or haplotypes, and the log rank test was applied to compare rejection curves. Cox regression analysis was used to assess the influence of patient characteristics (age, sex, ethnicity, year of transplantation, and underlying disease) and the numbers of haplotypes on the risk of acute rejection, using the time to the first episode

Table 2 Association between patient characteristics and time to first acute rejection, as analysed by Cox regression and stratified on centre

| Patient characteristic	HR (95% CI)	p Value
Sex		0.04
Male (n = 259)	1	
Female (n = 224)	1.35 (1.01–1.79)	
Age (per year)	0.99 (0.98–1.01)	0.29
Year of transplantation (per year)	0.96 (0.91–1.03)	0.25
Ethnicity		0.10
Caucasian (n = 449)	1	
Black African (n = 19)	0.43 (0.13–1.36)	
Asian (n = 15)	0.41 (0.10–1.65)	
Diagnosis		0.01
Chronic viral hepatitis (n = 94)	1	
Autoimmune related (n = 172)	2.06 (1.25–3.39)	
Alcoholic cirrhosis (n = 80)	1.37 (0.77–2.44)	
FHF (n = 44)	2.37 (1.29–4.34)	
Other (n = 93)	1.79 (1.04–3.09)	

Groups with a hazard ratio (HR) of 1 were chosen as the reference categories. 95% CI, 95% confidence interval.
p values were derived from the log likelihood test.
FHF, fulminant hepatic failure.

of acute rejection as outcome. To adjust the contributions of genetic CTLA-4 haplotypes for those of patient characteristics to the risk of acute rejection, multiple Cox regression analysis was performed. In all Cox regression analyses patients were stratified on centre to correct for the influence of the different centres on outcome. A p value of <0.05 was considered statistically significant.

RESULTS

Patient characteristics and acute rejection

The incidence of acute rejection in the investigated cohort was 39% (189 out of 483 patients). Table 2 shows the influence of demographic patient characteristics on the rate of acute rejection. Risk ratios of acute rejection were calculated by Cox regression using the time to the first acute rejection episode as outcome and stratifying patients on centre. Female recipients had a higher incidence of rejection compared with male recipients. Patients with a viral liver infection (either HCV or HBV) as the indication for liver transplantation had about a twofold decreased risk of acute rejection compared with patients with other underlying liver diseases, except alcoholic cirrhosis. Recipient age and year of
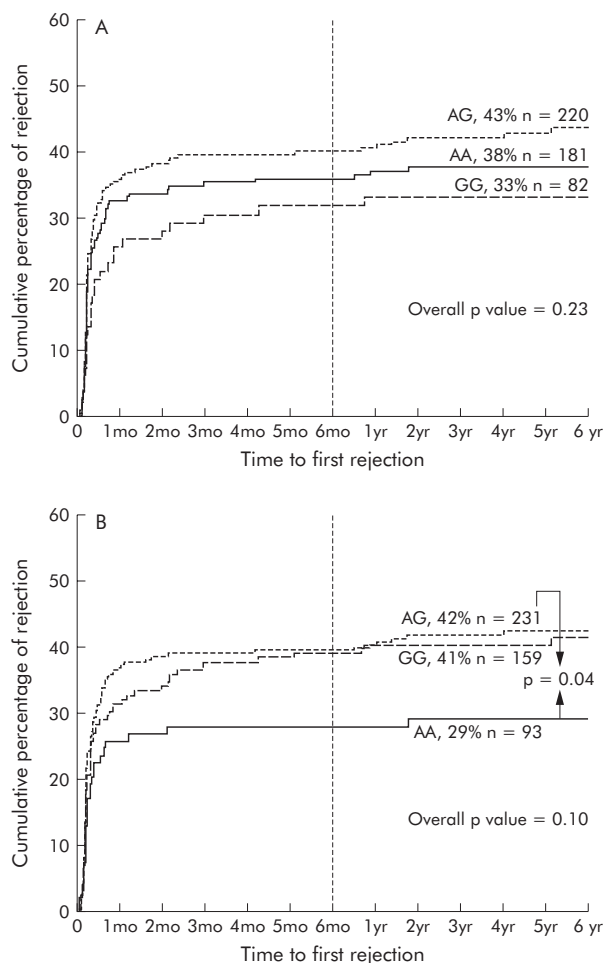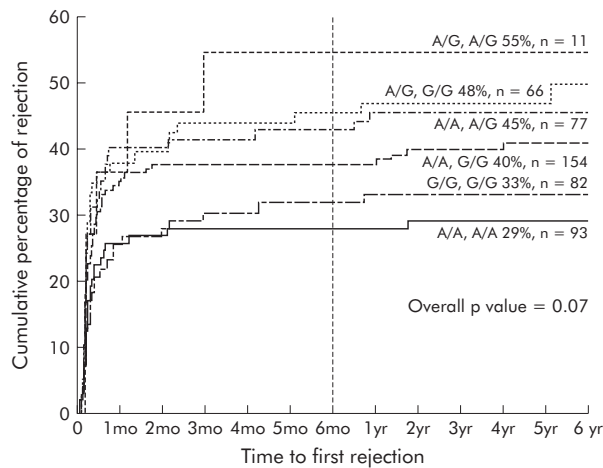

Figure 1 Cumulative percentages of acute rejection (Kaplan-Meier curves) in liver transplant recipients classified according to their cytotoxic T lymphocyte antigen 4 (CTLA-4) +49A/G (A) or CTLA-4 +6230G/A (B) genotype. The outcome used was the time to the first episode of acute rejection. Percentages indicate cumulative incidences of acute rejection in each group, eight years after transplantation. For better visualisation of the kinetics of rejection, the x axis of the first half year was spread out and curves were cut off at six years. No acute rejections occurred after that time. p values were derived from the log rank test.
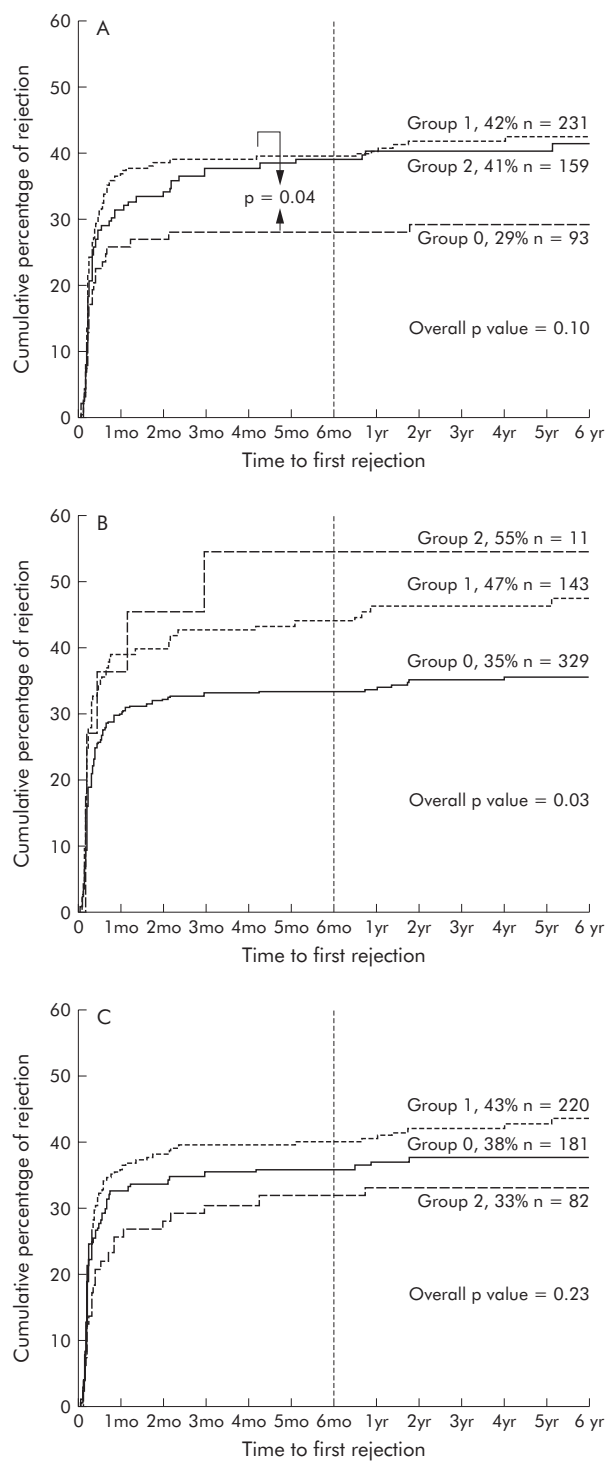
Figure 2 Cumulative percentages of acute rejection in liver transplant recipients classified according to the numbers of CTLA-4 +49/+6230 haplotype copies they carried. This is shown for haplotypes +49A/+6230A (A), +49A/+6230G (B), and +49G/+6230G (C). Patients who lacked the haplotype are termed group 0, heterozygous carriers group 1, and homozygous carriers group 2. The outcome used was the time to first episode of acute rejection. Percentages indicate cumulative incidences of acute rejection six years after transplantation. p values were derived from the log rank test.

transplantation were not significantly related to risk of acute rejection. The latter indicates that changes in immunosuppressive treatment over the years did not significantly influence the rate of acute rejection.

Figure 3 Cumulative percentages of acute rejection in liver transplant recipients classified according to their haplotype genotypes. Each curve represents a group of patients with one genotype, which is depicted above or underneath the curve with the +49/+6230 genotypes, separated by a comma. The outcome tested was the time to first episode of acute rejection. Percentages indicate cumulative incidences of acute rejection six years after transplantation. The p value is derived from the log rank test.

Single nucleotide polymorphisms and acute rejection

Figure 1 shows the cumulative probabilities of acute rejection in patient groups with different genotypes of the +49A/G and +6230G/A SNPs. Overall, neither SNP was significantly associated with acute rejection but pairwise comparisons of the genotypes showed a significantly lower risk of rejection in patients with the +6230AA genotype compared with those with the AG genotype.

Haplotypes and acute rejection

The +49A/G and +6230G/A SNPs were in complete linkage disequilibrium ($D' = 1$). Haplotype reconstruction from the SNP by Phase software showed that only three of the four possible haplotype combinations were present in the cohort—namely, +49A/+6230A, +49A/+6230G, and +49G/+6230G. To establish the associations between these haplotypes and acute rejection, we grouped patients by haplotype copy numbers (non-carriers, heterozygous carriers, and homozygous carriers) for each haplotype allele. Kaplan-Meier curves of the cumulative incidences of acute rejection in the different groups are depicted in fig 2. As the +6230A allele was completely linked to the +49A allele, the curves of the +49A/+6230A haplotype (fig 2A) were exactly the same

compared with the curves of the +6230G/A SNP (fig 1B), and the rate of rejection was significantly lower in patients homozygous for the +49A/+6230A haplotype compared with heterozygous carriers. However, the opposite haplotype +49G/+6230G was not a risk allele for acute rejection (fig 2C). Log rank analysis showed that overall only the +49A/+6230G haplotype (fig 2B) was significantly associated with the rate of acute rejection. To investigate whether the influence of this haplotype on the risk of rejection was dose dependent, a univariate Cox regression model was used in which the +49A/+6230G haplotype was introduced as a continuous variable. In this model, the +49A/+6230G haplotype demonstrated a significant dose-allele effect with a hazard rate of 1.39 (95% confidence interval 1.08–1.79; $p = 0.01$). Collectively, these data indicate that the CTLA-4 +49A/+6230G haplotype is a co-dominant risk allele for acute rejection after liver transplantation. When patients were classified according to their CTLA-4 +49/+6230 haplotype genotypes, Kaplan-Meier plots confirmed that homozygous and heterozygous carriers of the +49A/+6230G haplotype allele had the highest rates of acute rejection (fig 3), and homozygous carriers of both of the other haplotypes (49A/+6230A and +49G/+6230G) the lowest.

Multivariate analysis of the association of CTLA-4 haplotypes and patient characteristics with the rate of acute rejection

To determine whether the CTLA-4 haplotypes contributed to the risk of acute rejection independently of patients characteristics, a Cox regression model which included all haplotypes and patients characteristics was used. Non-significant parameters were removed stepwise. Table 3 shows that only the +49A/+6230G haplotype and underlying liver disease were significantly associated with rejection. Although in the univariate analysis a significant association between recipient sex and acute rejection was found, this was not the case in multivariate analysis. Adjusted for the effect of underlying diseases, the +49A/+6230G haplotype was dose dependently associated with acute rejection, with a risk ratio of 1.34 for each additional +49A/+6230G haplotype allele. This means that heterozygous carriers had a 1.34 higher relative risk of acute rejection compared with patients who lacked this haplotype, and that homozygous carriers had a 1.34 higher relative risk compared with heterozygous carriers.

DISCUSSION

In the present study, we analysed the influence of two functional SNPs in the CTLA-4 gene on the rate of acute rejection after liver transplantation. The +49A/G SNP is located in the signal peptide of the molecule and influences expression of the full length isoform on the T cell membrane.

Table 3 Multiple Cox regression analysis of the influence of cytotoxic T lymphocyte antigen 4 (CTLA-4) +49/+6230 haplotypes and patient characteristics stratified on centre on the rate of acute rejection

	Risk ratio	95% CI	p Value
Haplotype +49A/+6230G (per allele number)	1.34	1.04–1.72	0.02
Diagnosis			
Chronic viral hepatitis	1		0.01
Autoimmune related	2.05	1.24–3.35	<0.01
Alcoholic cirrhosis	1.34	0.75–2.39	0.33
FHF	2.25	1.23–4.14	0.01
Others	1.76	1.02–3.04	0.04

In the Cox regression model, each of the three different CTLA-4 +49/+6230 haplotypes were included as a continuous variable, together with underlying liver disease, year of transplantation, and recipient ethnicity, sex, and age. The diagnosis group with a hazard ratio of 1 was chosen as the reference category. Only those parameters which were significantly associated with acute rejection are shown. The overall p value was 0.003 (log likelihood test).
FHF, fulminant hepatic failure; 95% CI, 95% confidence interval.

Full length CTLA-4 molecules encoded by the G allele are incompletely glycosylated, leading to retrograde transport of a portion of the molecules to the cytoplasm for degradation. This results in reduced expression on the T cell surface[18] and impaired inhibitory function of CTLA-4 on T cell activation in individuals homozygous for +49G.[7] The +6230G/A SNP is located in the 3′ untranslated region of the CTLA-4 gene and was reported to influence the production rate of the soluble alternative splice form of CTLA-4. sCTLA-4 mRNA encoded by the +6230G-allele is produced at a reduced rate compared with mRNA encoded by the +6230 A allele.[11] As the soluble isoform is a suppressor of allogeneic T cell activation,[5] it is conceivable that carriers of the +6230G allele may be more susceptible to rejection after organ transplantation. This was indeed what we found on analysis of the association between the +6230 SNP and rejection: homozygous CTLA-4 +6230A liver transplant recipients which lacked the G allele showed a reduced rate of rejection compared with carriers of the G allele.

However, haplotype analysis showed that the single +6230G allele was not the risk allele for acute rejection. The +6230G allele was not associated with an increased risk of rejection when it was combined in a haplotype with the +49G allele, but only when it was combined with the +49A allele. Thus the risk allele for acute rejection after liver transplantation (+49A/+6230G) combines the genetic predisposition for reduced capability to produce sCTLA-4 (+6230G) with a normal capacity to express the full length CTLA-4 isoform on the T cell membrane (+49A). The effect of this haplotype on rejection was dose dependent, meaning that each additional allele results in a higher risk of rejection. The conclusion that this specific combination of single alleles confers risk to acute rejection is further substantiated by the observation that carriers of one +49A/+6230A allele and one +49G/+6230G allele had a rate of rejection intermediate between carriers of the +49A/+6230G risk haplotype and homozygous carriers of the protecting +49A/+6230A and +49G/+6230G haplotypes (fig 3). Conceivably, the single risk alleles +49A and +6230G can also exert their combined effect on rejection when they are located on different chromosomes.

The finding that only the +49A/+6230G haplotype is the risk allele for acute rejection is remarkable. The +49A/+6230G and +49G/+6230G haplotypes were observed to be associated to a similar extent with Graves' disease.[11] For type I diabetes, the +49G/+6230G is a risk allele but the association of the +49A/+6230G haplotype with disease is either positive or negative, depending on the allele of the −319C/T SNP which is cotransmitted.[12] It is difficult to imagine how increased expression of the inhibitory mCTLA-4, as encoded for by the +49A allele, contributes to an enhanced rate of rejection. A major difference between acute rejection of clinical organ grafts and autoimmune diseases is that rejection develops under the cover of continuous immunosuppression. Calcineurin inhibitors such as ciclosporin and tacrolimus inhibit the upregulation of mCTLA-4 expression on the cell surface after T cell activation.[19] As sCLTA-4 is produced by resting, but not by activated T cells, production of this isoform is probably not suppressed by calcineurin inhibitors. Therefore, the ratio between levels of mCTLA-4 and sCLTA-4 expression in organ transplant recipients may be quite different from that in patients with developing autoimmune diseases. As a consequence, the effects of high or low mCTLA-4 expression on acute rejection may differ from those on development of autoimmune diseases. Furthermore, an additional unknown single allele which influences CTLA-4 expression of function may be linked to the +49A+6230G haplotype, but not the +49G+6230G allele. The effect of this allele may only penetrate into a clinical effect under conditions of immunosuppression.

In a previous explorative single centre study, we found that liver transplant recipients who were homozygous for the CTLA-4 +49 G allele had a lower incidence of acute rejection after liver transplantation compared with homozygous or heterozygous carriers of the A allele.[16] A trend towards a similar association was found in the present study, but this was not statistically significant. Our previous finding seemed to contradict observations that the CTLA-4 +49G allele is a disease predisposing allele for several autoimmune diseases.[7–10] In addition, Slavcheva and colleagues[20] found no association between the +49 A/G polymorphism and acute rejection in a combined cohort of liver and kidney transplant recipients. The results of the present study may explain why differences in associations between the +49A/G polymorphism and acute rejection can be obtained in different study cohorts. Individuals with genotype +49G/G lack the risk haplotype allele +49A/+6230G, which makes them less susceptible to rejection, but carriers of +49A alleles can either carry the risk haplotype +49A/+6230G or the protective haplotype +49A/+6230A in conjunction with the +49A allele. Therefore, the association of the +49A/G genotype with rejection is dependent on the proportion of +49A alleles that are linked to the +6230G allele in the study cohort. In the present study, more than half (93/181) of the patients with the +49A/A genotype were homozygous carriers of the +49A/+6230A haplotype allele, and therefore had a low rate of acute rejection (fig 3). Consequently, in the cohort investigated in the present study, no significant association between the +49A/G polymorphism and rejection was found. The same may have been the case in the cohort studied by Slavcheva and colleagues.[20]

The results of the present study do not contradict the association between liver graft survival and recipient CTLA-4 +49 genotype observed by Marder and colleagues.[21] These authors reported that liver transplant recipients with the +49G/G genotype have a decreased graft survival in comparison with patients with +49A/A or A/G. The shortened graft survival was, however, not due to a higher incidence of acute rejection in this group but mainly related to recurrence of viral hepatitis. Therefore, the decreased graft survival in the +49G/G group was probably due to an increased immune response of the recipient to reinfection of the liver graft with hepatitis virus. Indeed, the CTLA-4 +49G allele has been associated with an enhanced immune response against HBV.[13]

The only patient characteristic that was significantly associated with acute rejection on multiple analysis was underlying liver disease. Liver transplant recipients with viral hepatitis or alcoholic cirrhosis as indication for liver transplantation had a twofold reduced risk of acute rejection. The first group included patients with HBV and HCV infection, transplanted for either acute liver failure or cirrhosis due to chronic infection. The reduced incidence of rejection is this patient category was probably due to prophylactic treatment of HBV positive patients with high dose anti-hepatitis B immunoglobulins to prevent reinfection of the graft. Recently, we confirmed the original observation of Farges and colleagues[22] that treatment with intravenous anti-hepatitis B immunoglobulins protects against rejection, and we demonstrated that these immunoglobulins inhibit both T cell and dendritic cell function.[23] The relatively low incidence of rejection in patients with alcoholic cirrhosis has also been observed in other studies.[24]

In conclusion, the CTLA-4 +49A/+6230G haplotype is a co-dominant risk allele for acute rejection after clinical liver transplantation. This implies that, even under immunosuppression, CTLA-4, in common with experimental animals, is critically involved in the regulation of the human immune response against allogeneic grafts.

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