

# Interleukin 10 and Tumor Necrosis Factor- $\alpha$ Genotypes in Rheumatoid Arthritis — Association with Clinical Response to Glucocorticoids

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**ABSTRACT. Objective.** There are dysregulated levels of interleukin 10 (IL-10) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in rheumatoid arthritis (RA), and their role in the disease is controversial. We analyzed the association of functional polymorphisms of IL-10 and TNF- $\alpha$  with susceptibility and disease characteristics at the time of diagnosis, and we also evaluated their possible use as predictors of clinical response to treatments.

**Methods.** Patients with recent-onset RA ( $n = 162$ ) and healthy controls ( $n = 373$ ) were genotyped for  $-1082$  IL-10 and  $-308$  TNF- $\alpha$  polymorphisms and data were related to clinical and immunological measurements of patients at the time of diagnosis. Response to treatment after 6 months was determined in 125 patients by the absolute change in Disease Activity Score (DAS28) and the American College of Rheumatology criteria for improvement.

**Results.** We found a reduced frequency of the low IL-10 producer genotype ( $-1082AA$ ) in patients with RA compared to controls (26.5% vs 38.9%;  $p = 0.006$ ), while it is a risk factor for anticyclic citrullinated peptide antibodies (anti-CCP) positivity ( $p = 0.028$ ). Evaluation of clinical response to treatments indicated that carriage of the high IL-10 genotype was associated with a favorable outcome ( $p = 0.009$ ), specifically to prednisone therapy ( $p = 0.0003$ ). No significant effects were observed with TNF- $\alpha$  polymorphism alone; however, in combination with the IL-10 genotype, it increased the strength of these associations.

**Conclusion.** Results show an association between the low IL-10 producer genotype and protection from RA; nevertheless, when other specific genetic and/or environmental factors trigger onset of RA, this genotype may predispose to development of anti-CCP+ RA disease with reduced response to prednisone treatment. (J Rheumatol First Release Jan 15 2010; doi:10.3899/jrheum.090566)

## Key Indexing Terms:

RHEUMATOID ARTHRITIS

CYTOKINES

POLYMORPHISMS

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CORTICOSTEROIDS

Rheumatoid arthritis (RA) is the most common autoimmune inflammatory rheumatic disorder. Although the etiology of the disease is unknown, genetic as well as environmental factors contribute to susceptibility and severity. The genetic

contribution is estimated to be 50%–60%<sup>1</sup>, with the HLA region having an important influence on genetic risk, in particular, HLA-DRB1 alleles encoding the shared epitope (SE) sequence. However, the contribution of the MHC represents no more than 30%–50% of the total genetic background<sup>2</sup>, pointing to the relevance of other susceptibility genes<sup>3</sup>. Several cytokines have been associated with the pathogenesis of RA and have played a regulator and effector role in the inflammatory response of this pathology<sup>4</sup>. Since the production of these molecules is controlled at the genetic level, functional polymorphism in their promoters could influence the development and severity of the disease. In particular, the production of interleukin 10 (IL-10) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), 2 mutually regulated cytokines involved in inflammatory responses, has been found to be dysregulated in patients with RA. Genetic polymorphisms at the promoter of both genes have been associated with different findings of cytokine production. The presence of the  $-1082G^*$  allele on the IL-10 gene and the

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–308A\* allele at the TNF- $\alpha$  promoter were associated with the highest basal and induced cytokine production<sup>5-8</sup>. Although these single-nucleotide polymorphisms (SNP) have been studied in patients with RA<sup>9-16</sup>, their role in the etiopathogenesis of the disease remains unclear owing to the conflicting results reported.

A central feature of RA is the presence of a number of autoantibodies, although only 2 systems are used in clinical practice: the rheumatoid factor (RF), usually of the IgM isotype and directed at the Fc region of IgG molecules, and the antibodies directed against cyclic citrullinated peptides (anti-CCP), more recently incorporated, but which are the most specific autoantibodies for RA<sup>17</sup>. In addition, anti-CCP antibodies may be detected years before disease onset<sup>18</sup>, are stable over time<sup>19</sup>, and are associated with joint destruction<sup>20,21</sup>. An increasing number of studies have supported the prognostic potential of these autoantibodies and suggest that their presence at disease onset may predict radiological damage and disease severity<sup>18,19-21</sup>. However, little is known about the factors involved in their development.

After onset of RA, an important requisite for a favorable outcome is to achieve early suppression of inflammation. In clinical practice, however, a sizable proportion of patients fail to respond adequately to therapies. The identification of genetic predictors of treatment response would thus provide valuable clinical information, because they can be determined at the time of diagnosis, when therapeutic intervention has the potential to offer the greatest benefits. We analyzed the association of functional IL-10 and TNF- $\alpha$  polymorphisms with RA susceptibility and with clinical and immunological features of patients at the time of diagnosis, and we also evaluated their possible use as predictors of clinical response to treatments.

## MATERIALS AND METHODS

**Patients.** The study population included 162 patients with RA (116 women and 46 men; mean age  $57.25 \pm 15.01$  yrs) consecutively recruited from the Early Arthritis Diagnosis outpatient clinic of the Hospital Central de Asturias, a unit that treats patients with RA for 3 years from the start of symptoms (median duration of disease at the time of sampling was 6 months). All patients were diagnosed with RA according to the American College of Rheumatology (ACR) criteria<sup>22</sup>. Clinical and laboratory results at the time of diagnosis and during the followup period were recorded. The healthy control group consisted of 373 unrelated blood donors (214 women and 159 men; mean age  $49.76 \pm 12.21$  years). All patients and controls were of Caucasian origin. Approval for the study was obtained from the regional Ethics Committee for Clinical Investigation and all determinations were performed with fully informed written consent, the anonymity of the data being guaranteed.

**Promoter polymorphism genotyping.** DNA was obtained from peripheral blood cells of patients and controls by standard procedures. SNP at positions –1082 on the IL-10 gene and –308 on the TNF- $\alpha$  gene were determined after amplification and hybridization with fluorescent-labeled probes (LightCycler, Roche Diagnostics, Mannheim, Germany), as reported<sup>7</sup>. The primers used were 5'-ATC CAA GAC AAC ACT ACT AAG GC and 5'-ATG GGG TGG AAG AAG TTG AA for –1082 IL-10 and 5'-CCT GCA TCC TGT CTG GAA GTT A and 5'-CTG CAC CTT CTG TCT CGG TTT for –308 TNF- $\alpha$ . The hybridization probes (designed by TIB MOLBIOL,

Berlin, Germany) were GGA TAG GAG GTC CCT TAC TTT CCT CTT ACC-F and LC Red 640-CCC TAC TTC CCC CTC CCA AA for –1082 IL-10 and AAC CCC GTC CCC ATG CCC C-F and LC Red 640-CCA AAC CTA TTG CCT CCA TTT CTT TTG GGG AC for –308 TNF- $\alpha$ .

**Clinical and laboratory examinations.** Data from physical and laboratory examinations and radiographs of hands and feet at the time of diagnosis were recovered from the Early Arthritis Diagnosis clinic database. Clinical data were as follows: age at diagnosis; duration of morning stiffness; number of swollen and tender joints; patient's global status and pain, assessed by a horizontal visual analog scale, range 0–100 for global status and 0–10 for pain status; functional disability, evaluated using the Health Assessment Questionnaire, range 0 to 3; and Disease Activity Score 28 (DAS28), a validated composite index that included 28-joint counts. Laboratory evaluations included the presence of IgM RF ( $> 20$  KU/l) and anti-CCP2 antibodies ( $> 25$  U/ml), determined using commercial kits (Immunochemistry systems, Beckman Coulter, La Brea, CA, USA, and Immunoscan RA Anti-CCP kit, Euro-Diagnostica AB, Madeon, Sweden, respectively); quantification of erythrocyte sedimentation rate (ESR; mm/h) and C-reactive protein, measured using standard laboratory methods; and the presence of at least 1 SE allele on the HLA-DRB1 gene, determined in 147 patients by polymerase chain reactions using specific primers (Cyclerplate System Protrans HLA-DRB1\*, Protrans Medizinische, Hockenheim, Germany). To ascertain clinical response to treatments, DAS28 was also determined in 125 patients at the time of sample collection and 6 months later, calculating the absolute change in DAS28 ( $\Delta$ DAS28) as a measure of treatment effectiveness. In addition, the percentage of ACR criteria for improvement (0, 20%, 50%, or 70%) was assessed in these patients at the end of the 6-month followup period. Achieving ACR70 was considered a good response to treatment.

**Statistical analysis.** The SPSS 15.0 statistical software package (SPSS Inc., Chicago, IL, USA) was used for all calculations. Genotype frequencies were obtained by direct counting. Hardy-Weinberg equilibrium, tested by the chi-squared test, was confirmed by the control population, but a deviation was detected in patients and then evaluation of the data was performed using genotype frequencies. Genotype distribution between RA patients and controls was compared using  $3 \times 2$  contingency tables and the chi-squared test. Differences in the frequency of genotypes were assessed by unconditional logistic regression and determination of risk. Clinical, immunological, and genetic measurements of patients were compared between sexes and cytokine genotypes using the chi-squared test for categorical variables and the Kruskal-Wallis or Mann-Whitney U test and analysis of variance or the Student's t-test for continuous variables, after checking their normality by means of Kolmogorov-Smirnov tests. Linear regression analyses were performed to investigate the association between IL-10 genetic polymorphism and treatment response, defined as the absolute change in DAS28 after 6 months ( $\Delta$ DAS28). A multivariate model was subsequently applied to determine the influence of different treatments and genetic, immunologic, or demographic factors as possible predictors of clinical response ( $\Delta$ DAS28, dependent variable). Linear regression coefficient (B) and 95% confidence interval (CI) were used as an estimate of the association. Finally, the effect of immunogenetic measurements and clinical features on the presence of anti-CCP antibodies at RA diagnosis (dependent variable) and the association between good response to prednisone treatment (achieving an ACR70) and cytokine genotypes was determined by univariate binary logistic regression followed by a multivariate analysis (backward logistic regression modeling) to define the effect of other measurements. For the variable stepwise selection process, p values  $< 0.05$  at entry and  $\geq 0.1$  for removal and classification cutoff of 0.5 were used. Estimation terminated when measurement estimates changed by  $< 0.001$ . OR and 95% CI were used as an estimate of the risk. The level of significance was set at  $p < 0.05$  for all analysis.

## RESULTS

*Association of cytokine genotypes with RA susceptibility and*

*clinical features at diagnosis.* Clinical and laboratory characteristics of patients with RA at the time of diagnosis are shown in Table 1. The distribution of the -1082 IL-10 and -308 TNF- $\alpha$  SNP in the population with RA was compared with that in healthy controls (Table 2), revealing significant differences in IL-10 genotypes ( $3 \times 2$  contingency tables and chi-squared test,  $p = 0.007$ ). Further, classification into low and high producer genotypes, as reported<sup>5,6</sup>, showed a significantly lower frequency of the low IL-10 producer genotype in patients with RA. The deviation of genotype frequencies from Hardy-Weinberg proportion observed in patients can provide additional evidence for the association between RA and IL-10 SNP<sup>23</sup>. No significant differences between patients and controls were observed in the frequency of -308 TNF- $\alpha$  polymorphism.

We subsequently investigated clinical and immunological characteristics at diagnosis of patients with RA. Some authors have reported gender differences in the disease phenotype among patients with RA; however, except for older age at diagnosis in men ( $p = 0.029$ ), no significant gender differences were noted at diagnosis (Table 1). Moreover, comparison of clinical features between patients with different IL-10 genotypes (Table 3) showed that -1082GG patients presented later diagnosis (at age  $61.25 \pm 18.40$  yrs), but separate analysis by sexes indicated that this association was present in men (GG:  $70.71 \pm 4.68$  yrs vs AA/AG:  $57.89 \pm 14.06$  yrs;  $p = 0.022$ ), but not in women ( $56.15 \pm 21.09$  vs  $53.88 \pm 14.40$  yrs;  $p =$  nonsignificant). With the exception of slightly higher ESR levels in -1082GG patients, clinical measurements at diagnosis did not show significant differences between IL-10 genotypes. However, studies of immunological features showed a trend toward fewer autoantibodies, particularly anti-CCP, in patients with the

*Table 2.* Distribution of IL-10 and TNF- $\alpha$  genotypes in patients with RA and healthy controls. Differences between patients and controls were evaluated by contingency tables and chi-square test.

Genotype	Controls, n = 373 n (%)	Patients, n = 162 n (%)	p
-1082 IL-10			
AA	145 (38.9)	43 (26.5)	
AG	174 (46.6)	99 (61.1)	0.007
GG	54 (14.5)	20 (12.3)	
Low (AA)	145 (38.9)	43 (26.5)	0.006*
High (GG/GA)	228 (61.1)	119 (73.5)	
-308 TNF- $\alpha$			
AA	8 (2.1)	0	
AG	77 (20.6)	30 (18.5)	0.136
GG	288 (77.2)	132 (81.5)	
Low (GG)	288 (77.2)	132 (81.5)	0.270**
High (AA/GA)	85 (22.8)	30 (18.5)	

\* AA vs GG/GA,  $p = 0.006$ , OR (95% CI): 0.57 (0.38–0.85). \*\* GG vs AA/GA,  $p = 0.270$ , OR (95% CI): 1.30 (0.82–2.07).

-1082GG genotype. Further, separate analysis by sexes showed that this trend was present only in women (anti-CCP-positive, GG: 30.8%, GA: 55.4%, AA: 62.1%; RF-positive, GG: 46.2%, GA: 63.5%, AA: 62.1%), suggesting that the -1082 IL-10 genotype might be involved in the generation of anti-CCP antibodies in female patients with RA. No significant differences were observed in clinical or immunological features at diagnosis among patients with different TNF- $\alpha$  genotypes.

*Association between cytokine genotypes and presence of anti-CCP antibodies at diagnosis.* The presence of anti-CCP antibodies before appearance of the first clinical symptoms of RA has been reported<sup>18</sup>, suggesting their predictive value

*Table 1.* Patient characteristics at RA diagnosis. Values are the mean  $\pm$  SD except for number of swollen and tender joints, which are median (range), and RF, anti-CCP, and presence of HLA-DRB1, which are n (%). Differences between women and men were evaluated using the chi-square test for categorical variables and Mann-Whitney U test or Student t test for continuous variables.

Characteristics	All Patients, n = 162	Women, n = 116	Men, n = 46
Age at RA diagnosis (yrs)	55.76 $\pm$ 15.01	54.14 $\pm$ 15.20	59.84 $\pm$ 13.84*
DAS28	5.85 $\pm$ 0.043	5.78 $\pm$ 0.942	6.01 $\pm$ 1.26
Number of swollen joints	6.0 (0–23)	6.0 (0–22)	8.0 (2–23)
Number of tender joints	10.0 (0–27)	10.0 (0–25)	12.0 (3–27)
Duration of morning stiffness (min)	105.93 $\pm$ 86.86	101.28 $\pm$ 86.10	118.00 $\pm$ 88.69
Global patient assessment	56.66 $\pm$ 21.89	56.69 $\pm$ 20.66	56.56 $\pm$ 25.06
Patient pain assessment	6.10 $\pm$ 2.08	6.13 $\pm$ 2.16	6.02 $\pm$ 1.89
HAQ score	1.37 $\pm$ 0.66	1.36 $\pm$ 0.65	1.40 $\pm$ 0.72
CRP (mg/dl)	6.57 $\pm$ 18.32	6.16 $\pm$ 19.40	7.64 $\pm$ 15.35
ESR (mm/h)	41.96 $\pm$ 26.01	40.75 $\pm$ 24.24	45.07 $\pm$ 30.19
Presence of HLA-DRB1 SE	78 (53.1)	53 (50.0)	25 (61.0)
RF positivity	100 (61.7)	71 (61.2)	29 (63.0)
Anti-CCP positivity	90 (55.6)	63 (54.3)	27 (58.7)

\*  $p = 0.029$ . DAS28: Disease Activity Score 28; HAQ: Health Assessment Questionnaire; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor; CCP: cyclic citrullinated peptides.

**Table 3.** Clinical and immunological features at diagnosis in patients with RA, grouped by –1082 IL-10 genotype. Values are the mean  $\pm$  SD except for number of swollen and tender joints, which are median (range), and sex, presence of RF, anti-CCP, and SE in HLA-DRB1, which are n (%). Differences between genotypes were evaluated using the chi-square test for categorical variables and Mann-Whitney U test or Students t test for continuous variables.

	–1082 IL-10 Genotype		
	AA, n = 43	AG, n = 99	GG, n = 20
Age at diagnosis, yrs	54.11 $\pm$ 14.13	55.36 $\pm$ 14.54	61.25 $\pm$ 18.40*
Sex			
Women	29 (67.4)	74 (74)	13 (65)
Men	14 (32.6)	25 (25.3)	7 (35)
DAS28	5.62 $\pm$ 1.04	5.90 $\pm$ 0.97	6.05 $\pm$ 1.30
Number of swollen joints	5.0 (0–18)	6.0 (1–22)	7.5 (0–23)
Number of tender joints	10.0 (1–25)	11.0 (2–27)	9.0 (0–26)
Duration of morning stiffness (min)	105.08 $\pm$ 86.12	103.97 $\pm$ 85.59	116.55 $\pm$ 97.47
Global patient assessment	54.60 $\pm$ 21.35	57.06 $\pm$ 22.01	58.85 $\pm$ 23.17
Patient pain assessment	5.85 $\pm$ 2.07	6.19 $\pm$ 2.06	6.20 $\pm$ 2.26
HAQ score	1.22 $\pm$ 0.60	1.39 $\pm$ 0.63	1.60 $\pm$ 0.86
CRP (mg/dl)	4.42 $\pm$ 11.38	6.45 $\pm$ 18.96	11.80 $\pm$ 25.79
ESR (mm/h)	35.32 $\pm$ 22.88	42.56 $\pm$ 24.81	53.30 $\pm$ 34.07**
SE positivity	21 (55.3)	49 (54.4)	8 (42.1)
RF positivity	26 (60.5)	63 (63.6)	11 (55.0)
Anti-CCP positivity	24 (55.8)	57 (57.6)	9 (45.0)

\* GG vs AA/AG;  $p = 0.036$ . \*\* GG vs AA;  $p = 0.041$ . DAS28: Disease Activity Score 28; HAQ: Health Assessment Questionnaire; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; SE: shared epitope; RF: rheumatoid factor.

and the relevance of knowing possible factors involved in their appearance. Thus we categorized women with RA into anti-CCP-positive and -negative groups at the time of diagnosis, and we used a logistic regression model to determine the possible association of genetic factors or clinical features at diagnosis with the presence of anti-CCP antibodies (Table 4). Results of the multivariate analysis (backward logistic regression modeling) indicated that early onset and presence of the –1082A\* allele on the IL-10 promoter (low producer) were risk factors for appearance of anti-CCP. Although no significant effect was observed with –308 TNF- $\alpha$  SNP alone, carriage of the combined genotype indicative of the highest IL-10 production (high IL-10/low TNF- $\alpha$ : –1082 GG/–308 GG) exerted a significant protective effect (OR 0.13, 95% CI 0.02–0.69,  $p = 0.017$ ). No association was found in men (data not shown).

**IL-10 genotypes and clinical response to treatments.** To evaluate the clinical effectiveness of treatment, DAS28 was determined in 125 patients with RA before and after 6 months of treatment, and the difference between initial and final DAS28 values ( $\Delta$ DAS28) was used to measure clinical response. In addition, we determined the percentage of ACR criteria for improvement (0, 20%, 50%, or 70%) at the end of the 6-month period. Patient stratification by IL-10 genotype showed no significant differences in disease activity (initial DAS28) before the followup period ( $p = 0.542$ , analysis of variance test). However, linear regression analysis showed an association between the IL-10 genotype and  $\Delta$ DAS28 (univariate regression coefficient 0.532, 95% CI

0.038–1.027,  $p = 0.035$ ), with carriage of the –1082G\* allele being a predictor of good response. To determine the possible influence of specific treatments and genetic, immunologic, or demographic factors on clinical response, a multivariate linear regression model was employed (Table 5). Results showed a significant influence of IL-10 alleles on clinical response ( $p = 0.009$ ) after adjustment for the treatments followed during the period evaluated and for genetic, immunologic, and demographic factors. In addition to IL-10 SNP, a slight positive association with low producer TNF- $\alpha$  genotype and male sex was detected, so that patients with the combined genotype containing the –1082G\* allele had the highest  $\Delta$ DAS28 levels (–1082GG/–308GG,  $n = 13$ :  $1.21 \pm 2.1$ ; –1082AG/–308GG,  $n = 63$ :  $0.75 \pm 1.62$ ; and –1082AA/–308GG,  $n = 27$ :  $0.56 \pm 1.63$ ). However, the most interesting result was that the use of prednisone was independently associated with a better DAS28 response ( $p = 0.0003$ ).

Finally, to corroborate that a combination of IL-10/TNF- $\alpha$  polymorphisms could be useful to predict the effectiveness of prednisone treatment, we analyzed clinical response by evaluating the percentage of ACR criteria for improvement. For this purpose, we considered patients achieving ACR70 as good responders to therapy. We accordingly categorized patients with prednisone treatment ( $n = 98$ , mean dose  $\pm$  SD,  $5.26 \pm 1.81$  mg/day), either alone or in combination with other agents, into 2 groups (ACR70 and ACR  $\leq$  50). Logistic regression analysis (Table 6) showed that carriers of the –1082G\* allele and the –308GG genotype (high

**Table 4.** Clinical and immunogenetic factors associated with presence of anti-CCP antibodies at diagnosis in women with RA. Values are the mean  $\pm$  SD except for number of swollen and tender joints, which are median (range), and sex, presence of RF, anti-CCP, and SE in HLA-DRB1, which are %. Differences were evaluated by logistic regression analysis using the presence of anti-CCP antibodies as dependent variable.

	Anti-CCP		Univariate Analysis		Multivariate Analysis*	
	Positive, n = 63	Negative, n = 53	OR (95% CI)	p	OR (95% CI)	p
Age at RA diagnosis, yrs	51.79 $\pm$ 16.05	56.92 $\pm$ 13.75	0.98 (0.95–1.00)	0.072	0.96 (0.92–0.99)	0.012
DAS28 score	5.79 $\pm$ 0.86	5.78 $\pm$ 1.04	1.01 (0.68–1.51)	0.969		
Number of swollen joints	6.00 (0–18)	5.00 (1–22)	0.98 (0.90–1.07)	0.662		
Number of tender joints	10.00 (0–25)	10.00 (2–24)	0.96 (0.89–1.03)	0.231		
HAQ score	1.37 $\pm$ 0.66	1.35 $\pm$ 0.63	1.03 (0.57–1.87)	0.912		
CRP (mg/dl)	5.33 $\pm$ 16.62	7.15 $\pm$ 22.38	1.00 (0.98–1.01)	0.618		
ESR (mm/h)	42.71 $\pm$ 24.35	38.43 $\pm$ 24.12	1.01 (0.99–1.02)	0.343		
HLA-DRB1 SE						
Presence	29 (50.9)	24 (49.0)	1			
Absence	28 (49.1)	25 (51.0)	0.93 (0.43–1.99)	0.846		
–1082 IL10 genotype						
GG	4 (6.3)	9 (17.0)	1		1	
AA + AG	59 (93.7)	44 (83.0)	3.02 (0.87–10.43)	0.081	5.15 (1.21–21.97)	0.027
–308 TNF genotype						
GG	54 (85.7)	42 (79.2)	1			
AA + AG	9 (14.3)	11 (20.8)	0.64 (0.24–1.68)	0.361		
Combined IL-10/TNF- $\alpha$ genotype						
AA+AG/GG	51 (81.0)	34 (64.2)	1		1	
AA+AG/AA+AG	8 (12.7)	10 (18.9)	0.53 (0.19–1.49)	0.230	0.66 (0.19–2.18)	0.493
GG/GG	3 (4.8)	8 (15.1)	0.25 (0.06–1.01)	0.052	0.13 (0.02–0.69)	0.017
GG/AA+AG	1 (1.6)	1 (1.9)	0.67 (0.04–11.02)	0.777	0.92 (0.04–19.16)	0.958

\* Backward logistic regression modeling. The variables entered in the initial model were the clinical and genetic measurements included in the table. The accuracy of the final prediction model was 71.0% for the analysis of single genotypes and 73% for the analysis of combined genotypes. CCP: cyclic citrullinated peptides; DAS28: Disease Activity Score 28; HAQ: Health Assessment Questionnaire; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; SE: shared epitope.

IL-10/low TNF- $\alpha$  producers) are the best responders to this treatment compared with patients with other combined genotypes. This association remained highly significant ( $p = 0.001$ ) in the multivariate analysis (backward logistic regression modeling) after adjustment for prednisone dose, sex, and presence of anti-CCP antibodies.

## DISCUSSION

Given the evident advantages of the use of genetic markers to predict the risk of disease onset and outcome, clarification of the role of IL-10 and TNF- $\alpha$  functional polymorphisms in RA remains a matter of considerable interest. We showed a reduced frequency of the low IL-10 producer genotype (–1082AA) in patients with RA, while it appears to be a risk factor for anti-CCP development. In accord with our results, this genotype was found to be underrepresented in patients with RA from a Polish and a Turkish population<sup>9,10</sup>. Other authors did not observe differences in the distribution of IL-10 genotypes<sup>11–13,16</sup>, while a high prevalence of the low IL-10 producer genotype was reported in a Swedish population with RA, although in that study genotype frequencies from healthy controls were significantly different from ours<sup>14</sup>. As regards the TNF- $\alpha$  genotype, no significant differences were found between patients and controls. Indeed, in spite of the important role that has been attributed to this cytokine in RA, the absence of a significant association of the –308

TNF- $\alpha$  SNP with disease susceptibility has been described by most authors<sup>10,15</sup>.

IL-10 levels have frequently been found to be increased in patients with RA<sup>24</sup>; however, the role played by this cytokine is still controversial. Although IL-10 is usually considered to mediate potent downregulation of the inflammatory responses, its ability to enhance systemic inflammation and to increase the production of proinflammatory molecules has also been reported<sup>25</sup>. As a B cell stimulator, its role in autoimmunity has been traditionally focused on its potential to generate antibody-producing cells, thereby contributing to humoral immunity. In recent years, however, there has been growing interest in the contribution of antibody-independent functions of B cells to autoimmune responses. Indeed, memory B cells produce proinflammatory cytokines<sup>26</sup> and are responsible for an ectopic lymphoid neogenesis in the rheumatoid synovium, which was not associated with local production of RF or anti-CCP antibodies<sup>27</sup>. It has been reported that high IL-10 producer genotypes are increased in patients with a higher rate of joint destruction<sup>28</sup> and are associated with more severe radiographic damage in patients who are anti-CCP-negative and RF-negative<sup>29</sup>, thus supporting a detrimental effect of IL-10 unrelated to antibody production in patients with RA.

However, the finding of an association between the –1082A\* allele and the presence of anti-CCP antibodies at

*Table 5.* Factors predicting response to treatment after 6 months defined as change in DAS28 ( $\Delta$ DAS28). Multivariate linear regression analysis using  $\Delta$ DAS28 as the dependent variable and adjusted for treatments followed during the 6-month followup period and demographic, genetic, and immunologic factors included in the table.  $R^2 = 0.260$ .

Predictor of Clinical Response	n (%)	$\Delta$ DAS28, mean $\pm$ SD	Multivariate Linear Regression Coefficient (95% CI)	p
Age at diagnosis			-0.009 (-0.032-0.015)	0.466
Sex				
Women	88 (70.4)	0.43 $\pm$ 1.50	0.734 (0.038-1.431)	0.039
Men	37 (29.6)	1.04 $\pm$ 2.06		
-1082 IL-10 genotype				
AA	35 (28.0)	0.30 $\pm$ 1.69	0.647 (0.169-1.126)	0.009
AG	74 (59.2)	0.63 $\pm$ 1.63		
GG	16 (12.8)	1.18 $\pm$ 1.98		
-308 TNF- $\alpha$ genotype				
GA/AA	22 (17.6)	-0.09 $\pm$ 1.63	0.895 (0.040-1.750)	0.040
GG	103 (82.4)	0.76 $\pm$ 1.68		
HLA-DRB1 SE				
Negative	56 (49.6)	0.44 $\pm$ 1.71	-0.231 (-0.834-0.373)	0.450
Positive	57 (50.4)	0.68 $\pm$ 1.67		
RF				
Negative	47 (37.6)	0.33 $\pm$ 1.76	0.664 (-0.184-1.512)	0.123
Positive	78 (62.4)	0.78 $\pm$ 1.65		
Anti-CCP				
Negative	54 (43.2)	0.47 $\pm$ 1.71	-0.266 (-1.118-0.586)	0.537
Positive	71 (56.8)	0.71 $\pm$ 1.69		
Treatment				
Methotrexate				
Nonusers	27 (21.6)	0.33 $\pm$ 1.46	-0.331 (-1.233-0.571)	0.468
Users	98 (78.4)	0.69 $\pm$ 1.76		
(mean dose 18.09 $\pm$ 6.26 mg/week)				
Prednisone				
Nonusers	27 (21.6)	-0.09 $\pm$ 0.91	1.562 (0.732-2.391)	0.0003
Users	98 (78.4)	0.80 $\pm$ 1.81		
(mean dose 5.56 $\pm$ 1.59 mg/day)				
Leflunomide				
Nonusers	74 (59.2)	0.86 $\pm$ 1.71	-0.718 (-1.442-0.006)	0.052
Users	51 (40.8)	0.25 $\pm$ 1.63		
(mean dose 16.11 $\pm$ 4.92 mg/day)				
Etanercept				
Nonusers	119 (95.2)	0.60 $\pm$ 1.73	-0.475 (-1.965-1.015)	0.528
Users	6 (4.8)	0.85 $\pm$ 0.83		
Adalimumab				
Nonusers	121 (96.8)	0.63 $\pm$ 1.72	-0.583 (-2.488-1.322)	0.545
Users	4 (3.2)	0.03 $\pm$ 0.72		
Chloroquine				
Nonusers	124 (99.2)	0.61 $\pm$ 1.70	-2.565 (-5.880-0.750)	0.128
Users	1 (0.8)	-0.2		

DAS28: Disease Activity Score 28; TNF: tumor necrosis factor; SE: shared epitope; RF: rheumatoid factor; CCP: cyclic citrullinated peptide.

RA diagnosis could be of additional pathogenetic interest. Although this is the first study reporting this association, a relationship between genotypes encoding low IL-10 production and autoantibody appearance has been reported in various diseases, such as antineutrophil cytoplasmic antibodies in ulcerative colitis<sup>30</sup>; anti-SSA, anti-SSB, and anti-Sm antibodies in systemic lupus erythematosus<sup>7,31</sup>; antitransglutaminase antibodies in celiac disease<sup>32</sup>; and RF in RA<sup>16,33</sup>. In spite of the stimulatory effect of IL-10 on B

cells, these results could be explained in the context of an autoimmune inflammatory disease by the fact that low IL-10 levels do not allow efficient control of local inflammation, favoring tissue destruction and autoantigen exposition. Given that IL-10 and TNF- $\alpha$  are 2 mutually regulated cytokines that exert complex and predominantly opposite roles in inflammatory responses, it is not surprising that the strength of this association increases when the low IL-10 genotype is combined with high TNF- $\alpha$  production

Table 6. Association between IL-10/TNF- $\alpha$  genotypes and response to prednisone treatment. Association was calculated by unconditional logistic regression modeling using good response to prednisone treatment (achieving an ACR70) as the dependent variable.

Combined IL-10/ TNF- $\alpha$ genotype	ACR $\leq$ 50, n = 66	ACR 70, n = 32	Univariate Analysis OR (95% CI)	p	Multivariate Analysis* OR (95% CI)	p
-1082GG+GA/-308GG (high IL-10/low TNF- $\alpha$ )	30 (45.5)	25 (78.1)	4.286 (1.63–11.28)	0.003	10.30 (2.73–38.84)	0.001
Other combined genotypes	36 (54.5)	7 (21.9)	1		1	

\* Backward logistic regression modeling. The variables entered in the initial model were prednisone dose, other treatments used in combination with prednisone (methotrexate, leflunomide, etanercept, adalimumab), and genetic (HLA-DRB1 SE), immunologic (RF and anti-CCP antibodies), and demographic (sex and age) factors. The final model (accuracy of 77.3%) included prednisone dose, sex, and presence of anti-CCP antibodies. TNF: tumor necrosis factor; ACR: American College of Rheumatology; SE: shared epitope; RF: rheumatoid factor; CCP: cyclic citrullinated peptides.

(-308A\* allele). TNF- $\alpha$  is a proinflammatory and proapoptotic molecule clearly involved in the pathology of RA. It is thus reasonable to assume that increased local synthesis of this cytokine, which cannot be counterbalanced by the low production of IL-10, may create a microenvironment that increases these effects, thus perpetuating autoantigen exposure and an autoimmune inflammatory response.

Nonetheless, the most relevant clinical finding of our study was a significant association between carriage of the high IL-10 producer genotype and good response to prednisone treatment, the low TNF- $\alpha$  genotype once more presenting a slight effect. Glucocorticoids are powerful anti-inflammatory agents and low-dose steroid treatment has been reported to play an effective role in clinical and radiographic outcomes in RA<sup>34,35</sup>. Indeed, prednisone is usually given, either alone or in combination with disease-modifying antirheumatic drugs, at the start of RA. However, a significant proportion of patients fail to respond adequately to corticosteroid therapy<sup>36</sup>. This association, not previously reported in patients with RA, supports the use of these genetic markers as a predictor of glucocorticoid response, as suggested by other authors. Thus, in accord with our results, carriage of the -1082 AA genotype (low IL-10 producer) has been found to be a relevant risk factor for developing steroid dependency in inflammatory bowel disease<sup>37</sup> and in pediatric heart transplant patients<sup>38</sup>, while childhood acute lymphoblastic leukemia patients with the IL-10 GG genotype presented a protective effect from an initial poor response to prednisone<sup>39</sup>. To explain these results, it has been proposed that the upregulation of IL-10 production may be one mechanism by means of which steroids exert their beneficial effects. Increased levels of IL-10 following steroid administration are well documented<sup>40,41</sup>. In addition, it has been shown that IL-10 may increase sensitivity to glucocorticoids through upregulation of glucocorticoid receptor alpha expression, while TNF- $\alpha$  decreases it<sup>42</sup>. Moreover, IL-10 inhibits expression of the macrophage migration inhibitory factor<sup>43</sup>, which is increased in patients with RA<sup>44</sup> and which downregulates the immunosuppressive effects of corticosteroids<sup>45</sup>. Obviously, we cannot discard the effect of

other proposed molecular mechanisms contributing to impaired sensitivity to steroids, such as increased expression of the beta isoform of the glucocorticoid receptor<sup>46</sup>, overexpression of the multidrug resistance gene<sup>47</sup>, or excessive constitutive activation of the proinflammatory molecule nuclear factor- $\kappa$ B<sup>48</sup>.

Our results suggest that carriage of the low IL-10 producer genotype may protect for onset of RA; nevertheless, when other specific genetic and/or environmental factors trigger RA onset, this genotype may predispose to develop anti-CCP-positive RA disease with reduced response to treatment. Indeed, the combined low IL-10/high TNF- $\alpha$  genotype has also been correlated with reduced response to TNF- $\alpha$  blockers<sup>49,50</sup>. Moreover, the presence of anti-CCP antibodies (associated with this genotype) has been related to poor prognosis and reduced response to anti-TNF- $\alpha$  drugs<sup>51</sup>. All these results support that RA is a heterogeneous disease that could involve different etiopathogenic factors. Thus, treatments and management of the disease might be different depending on IL-10 and TNF- $\alpha$  genotypes and the presence of anti-CCP antibodies.

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