Relation of HLA-B27, Tumor Necrosis Factor-α Promoter Gene Polymorphisms, and T Cell Cytokine Production in Ankylosing Spondylitis — A Comprehensive Genotype-Phenotype Analysis from an Observational Cohort

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ABSTRACT. ***Objective.*** In a pilot study, a distinct T cell cytokine pattern associated with HLA-B27 status and a tumor necrosis factor-α (TNF-α) promoter gene polymorphism was found at –308 (TNF–308). The objective of our study was to assess these associations in a different cohort of patients with ankylosing spondylitis (AS) and to evaluate any effect on clinical measurements.

Methods. Peripheral T cell cytokine production of patients with AS (n = 121) from the German Spondyloarthritis Inception Cohort was assessed by flow cytometry and correlated with HLA-B27, TNF–238, and TNF–308, and with clinical measurements.

Results. In HLA-B27-positive, anti-TNF-naive patients with AS, the percentages of TNF-α-producing (5.02%) and interleukin 10-producing (0.31%) CD8+ cells were significantly lower in comparison to HLA-B27-negative patients (9.52%, p = 0.048, and 0.46%, p = 0.037, respectively). A nonsignificant trend was found for a lower production of TNF-α by CD4+ and interferon-γ by both CD4+ and CD8+ T cells, as compared to HLA-B27-negative patients with AS (p > 0.05 for all comparisons). The A allele at TNF–308 was associated with a lower percentage of TNF-α-producing CD4+ T cells. No significant correlations were found between clinical or radiological measurements and cytokine production or with TNF-α promoter gene polymorphisms.

Conclusion. Modulation of T cell cytokines by HLA-B27 might play a role in AS pathogenesis in B27-positive individuals. No conclusive data were obtained for the TNF–308 polymorphism on cytokine production, and no effect of cytokines or genetic polymorphisms on clinical manifestations was observed. (J Rheumatol First Release Sep 1 2011; doi:10.3899/jrheum.110130)

Key Indexing Terms:
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Ankylosing spondylitis (AS) is a chronic systemic inflammatory disease of unknown etiology that primarily affects the axial skeleton (sacroiliac joints and spine). The strong association between AS and the presence of the human leukocyte antigen (HLA) B27 has been established but the molecular mechanism behind this association remains unclear. We demonstrated a decreased proportion of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ)-producing T cells in both HLA-B27-positive patients with AS (n = 25) and HLA-B27-positive healthy subjects (n = 18) in comparison to HLA-B27-negative healthy controls (n = 22)[1]. This finding suggests a lower TNF-α and IFN-γ production by T cells as a potential disease susceptibility factor. The lower cytokine production may facilitate the survival and persistence of intracellular bacteria in HLA-B27-positive individuals, which may play a role not only in reactive arthritis but also in AS. Despite the strong association between AS and HLA-B27, AS develops only in a minority

(about 5%) of HLA-B27-positive subjects[2]. Twin studies demonstrated that HLA-B27 contributes < 40% of the genetic susceptibility to AS[3]. Therefore, efforts are continuing to identify other genes within and outside the major histocompatibility complex associated with AS and spondyloarthritis. A scan of 14,500 single-nucleotide polymorphisms revealed 2 new loci related to AS: ERAP1 (ARTS1) and interleukin (IL)-23R[4].

Another candidate is the TNF-α-encoding chromosomal area located within the HLA class III region, which also contains polymorphic sites. Several studies showed a lower frequency of the alternative allele A at positions –238 and –308 within the TNF-α promoter area in patients with AS in comparison to healthy controls[5,6,7,8,9]; however, in other reports there were no significant differences in allele distribution[10,11,12,13,14], or frequencies of alternative alleles were even higher in patients with AS[13,15]. Intriguingly, there are no conclusive data on the influence of TNF-α promoter polymorphisms on TNF-α production, nor on the influence of a certain genotype on the clinical presentation of AS. In our previous report we found a significantly higher percentage of TNF-α-positive T cells in HLA-B27-positive subjects carrying the A allele at the –308 position, but the number of patients carrying the alternative allele was small (n = 6: 4 healthy individuals and 2 patients with AS) and there were no data available from HLA-B27-negative patients with AS[1].

Our study aimed to assess any effect of both HLA-B27 and TNF-α promoter polymorphisms on T cell cytokine production of HLA-B27-positive and HLA-B27-negative patients with AS from a different cohort, and to explore the relation of these indications with clinical manifestations in these patients.

MATERIALS AND METHODS

Patients. In total, 121 patients with AS (79 men and 42 women) from the German Spondyloarthritis Inception Cohort (GESPIC) were analyzed. Of these, cytokine production could be analyzed in 107 patients, and TNF promoter gene polymorphisms in 84 and 81 patients, respectively, and both cytokine production and TNF polymorphisms in 70 patients. The design of GESPIC and its inclusion and exclusion criteria were reported elsewhere[16]. In brief, patients with AS had to fulfill the modified New York criteria[17] and had a maximum duration of AS symptoms ≤ 10 years. The mean age of the patients included in the current study was 35.3 ± 9.7 (range 18 to 76) years, the mean symptom duration was 5.9 ± 2.6 years, and the mean age at disease onset 29.3 ± 10.1 years. The majority (86%) of patients with AS were HLA-B27-positive. The median [interquartile range (IQR)] sacroiliitis grade (according to the modified New York criteria) was 3[2,3] for the right sacroiliac joint and 2.5[2,3] for the left sacroiliac joint. The median modified Stoke Ankylosing Spondylitis Spinal Score (mSASSS)[18] was 1.5 (0.5; 10.0). Treatment included nonsteroidal antiinflammatory drugs (63.6%), sulfasalazine (22.3%), glucocorticoids (6.6%), methotrexate (5.0%), and TNF-α antagonists (3.3%).

Disease activity and functional status were assessed using the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and the Bath Ankylosing Spondylitis Functional Index (BASFI), respectively. General pain and nocturnal pain levels were measured on a 0–10 numerical rating scale. The presence of peripheral arthritis, enthesitis (Berlin score), and uveitis, as well as spinal mobility (by means of the Bath Ankylosing Spondylitis Metrology Index, BASMI), were also assessed.

Ethical approval for the study and written informed consent from all patients were obtained.

Intracellular cytokine staining of peripheral blood T cells and analysis by flow cytometry. In order to compare the results with our earlier pilot study[1] we followed the same experimental protocol: peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) and frozen in liquid nitrogen until used. The detailed methodology of the intracellular cytokine staining and cell analysis has been described[1,19]. In brief, PBMC were thawed and 1×10^6 cells were cultured for 6 h in the presence of 5 ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma, St. Louis, MO, USA) and 1 ng/ml ionomycin (Sigma), with 2.5 μM monensin (Sigma) added during the last 2 h. The cells were then fixed, stained with monoclonal antibodies directed against cytokines (IL-4, IL-10, IFN-γ, TNF-α) and against the T cell surface markers CD3 and CD8, and subsequently analyzed by flow cytometry. CD4+ T cells were identified indirectly by gating on CD3+ but CD8– lymphocytes because PMA/ionomycin induces a downregulation of CD4 cell-surface molecules[1]. After gating either on CD3+/CD8+ or CD3+/CD8– (CD4) lymphocytes, data were analyzed with CELLQuest software and displayed as dot plots of FITC (x axis) and phycoerythrin (y axis) fluorescence (4 decade log scales). Quadrant markers were positioned to include > 99% of control immunoglobulin staining cells in the lower left quadrant.

Genotyping of TNF-α promoter polymorphisms. Genotyping of TNF-α promoter polymorphisms at positions –238 and –308 was performed using an amplification refractory mutation system polymerase chain reaction design, as described[1,20].

Statistics. All variables were tested for the distribution type using a 1-sample Kolmogorov-Smirnov test. The normal type of data distribution was considered if $p > 0.05$. Comparisons of normally distributed data were performed using the Student's t test for independent samples. In case of nonnormal distribution, the Mann-Whitney U test was applied for comparisons of independent samples. Normally distributed data are presented as mean ± SD. Nonnormally distributed data are presented as median (25th percentile; 75th percentile). Differences in frequencies were assessed by means of the chi-squared test. For correlation analysis, Spearman p coefficients were calculated. Statistical analysis was performed using SPSS 17.0 for Windows software (SPSS Inc., Chicago, IL, USA).

RESULTS

HLA-B27 status and T cell cytokine production. T cell (both CD4+ and CD8+) cytokine production was investigated in 92 HLA-B27-positive and 15 HLA-B27-negative patients. There was a statistically nonsignificant trend for lower percentages of TNF-α-producing CD4+ and CD8+ T cells in HLA-B27-positive patients with AS in comparison to HLA-B27-negative patients with AS. A similar nonsignificant trend was found for production of IFN-γ (Table 1).

No statistically significant differences in the percentages of IL-4-producing T cells (CD4+ and CD8+) and IL-10-producing CD4+ cells were found between HLA-B27-positive and HLA-B27-negative patients with AS (Table 1). The percentage of IL-10+ CD8+ T cells was lower in HLA-B27-positive patients in comparison to HLA-B27-negative patients [0.32% (0.18; 0.57) and 0.46% (0.34; 0.98), respectively; p = 0.019]. In a secondary analysis we excluded patients with AS treated with anti-TNF agents because anti-TNF treatment may influence T cell cytokine production[21,22]. The exclusion of anti-TNF-α-treated patients (n =

Table 1. Percentages of cytokine-producing T cells in HLA-B27-positive and HLA-B27-negative patients with AS; median (interquartile range) are shown.

T Cell	HLA-B27-positive, n = 92	HLA-B27-negative, n = 15	p
TNF-α+ CD4+	7.57 (4.32; 15.09)	10.43 (2.59; 21.05)	0.740
TNF-α+ CD8+	5.02 (2.64; 10.99)	6.89 (2.51; 29.79)	0.167
IFN-γ+ CD4+	5.45 (3.22; 8.93)	7.04 (2.82; 12.22)	0.536
IFN-γ+ CD8+	10.28 (5.74; 18.14)	14.54 (6.84; 29.21)	0.170
IL-4+ CD4+	0.42 (0.11; 0.92)	0.40 (0.05; 1.30)	0.771
IL-4+ CD8+	0.35 (0.14; 0.91)	0.56 (0.24; 1.39)	0.132
IL-10+ CD4+	0.42 (0.28; 0.72)	0.34 (0.23; 0.54)	0.319
IL-10+ CD8+	0.32 (0.18; 0.57)	0.46 (0.34; 0.98)	0.019

AS: ankylosing spondylitis; TNF: tumor necrosis factor; IFN: interferon; IL: interleukin.

4, 2 HLA-B27-positive and 2 HLA-B27-negative) from the analysis resulted in a statistically significant difference in TNF-α-producing CD8+ cells: 5.02% (2.57; 11.33) in HLA-B27-positive patients versus 9.52% (5.27; 29.85) in HLA-B27-negative patients (p = 0.048). The percentage of IL-10+ CD8+ T cells remained lower in HLA-B27-positive in comparison to HLA-B27-negative patients [0.31% (0.18; 0.58) and 0.46% (0.32; 0.86), respectively; p = 0.037]. All other trends remained unchanged.

TNF-α promoter polymorphisms, T cell cytokine production, and clinical manifestations of AS. The TNF-α promoter polymorphism at position –308 was investigated in 84 patients with AS and at position –238 in 81 patients.

At TNF–308 the alternative A allele occurred in 16 patients (19.1%); most of them (15) were heterozygous (GA genotype) and only 1 patient was homozygous (AA). As reported, the A allele at position –238 was rare and was found in only 4 patients with AS (4.9%); 2 patients had the GA genotype and 2 patients had the AA genotype. Alternative alleles at both positions were found to be more frequent in HLA-B27-positive patients than in HLA-B27-negative (Table 2), but differences were not statistically significant.

Genetic data on TNF-α polymorphisms and functional data related to T cell cytokine production were available for 70 patients. The alternative allele A at position –308 was detected in 14 individuals among these patients (all heterozygous: GA). The percentage (median, IQR) of CD4+ TNF-α-producing T cells (Figure 1A) was significantly lower in patients carrying the A allele (GA or AA) at position –308 (n = 14) than in homozygous GG (n = 56) patients [4.56% (2.76; 9.20) vs 10.45% (5.74; 15.09); p = 0.014]. There was a trend for a difference only in the median percentage of CD8+ TNF-α+ T cells (Figure 1B) between patients with GA and GG genotype [3.73% (1.88; 6.39) vs 6.72% (3.08; 12.94); p = 0.066]. Further stratification into HLA-B27-positive and HLA-B27-negative patients revealed significant differences in TNF-α production by CD4+ T cells between GA (n = 13) and GG (n = 47) carriers at TNF–308 in HLA-B27-positive patients [4.94% (2.85; 9.34) vs 10.47% (5.69; 15.20), respectively; p = 0.023], similar to the entire cohort. Among HLA-B27-negative patients, only 1 patient carried an A allele (median percentage of TNF-α-producing CD4+ T cells: 2.59%) and 9 patients had a GG genotype [10.43 (4.17; 21.81)]. At position –238, only 1 patient carried the A allele, therefore, any further analysis of the influence of the TNF–238 polymorphism on TNF-α production was not meaningful. The exclusion from the analysis of 4 anti-TNF-α-treated patients did not reveal meaningful differences compared to the above described results (data not shown).

No significant differences in clinical measurements (disease duration, age at onset, BASMI, BASFI, BASDAI, presence of peripheral arthritis, uveitis, enthesitis) or laboratory data [C-reactive protein (CRP), erythrocyte sedimentation rate (ESR)] were found between homozygous GG patients and patients carrying the alternative A allele (GA or AA) at the position –308, as well as at position –238 (data not shown). Further, the percentage of TNF-α, IFN-γ, IL-4, and IL-10-producing CD4+ and CD8+ T cells did not correlate with clinical or laboratory measurements (CRP, ESR, BASDAI, BASFI, BASMI), nor was it associated with clinical (peripheral arthritis, enthesitis, uveitis) or radiographic (sacroiliitis grade, mSASSS score) manifestations (data not shown).

Table 2. Distribution of the TNF-α promoter –238 and –208 alleles in HLA-B27-positive and HLA-B27-negative patients with AS.

TNF-α Promoter Polymorphism		HLA-B27-positive, n (%)	HLA-B27-negative, n (%)
–308	GG	57 (79.2)	11 (91.7)
	GA and AA	15 (20.8)	1 (8.3)
–238	GG	65 (94.2)	12 (100.0)
	GA and AA	4 (5.8)	0 (0.0)

TNF: tumor necrosis factor; AS: ankylosing spondylitis

DISCUSSION

We have described lower levels of TNF-α and IFN-γ-producing CD4+ and CD8+ T cells in HLA-B27-positive patients with AS (n = 25) and HLA-B27-positive healthy donors (n = 18) in comparison to HLA-B27-negative healthy persons (n = 22)[1]. It was speculated that the low production of TNF-α and IFN-γ by T cells in HLA-B27-positive persons as compared to HLA-B27-negative persons could increase the susceptibility to AS. One of the possible mechanisms could be an impaired elimination of bacteria, a situation that may play a role in the pathogenesis of AS.

We sought to assess this association among a cohort of 92 HLA-B27-positive and 15 HLA-B27-negative patients with AS from the GESPIC observational cohort, using the same

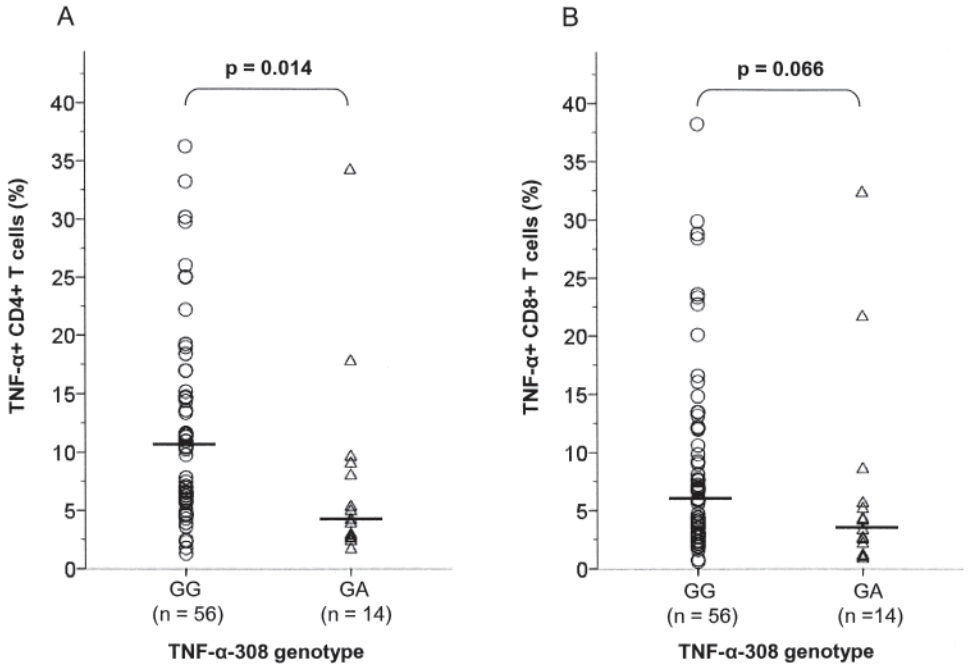

Figure 1. Percentages of tumor necrosis factor-α producing CD4+ (A) and CD8+ (B) T cells in relation to the TNF-α-308 genotype. Horizontal lines indicate medians.

established experimental protocol in the same laboratory as in the earlier pilot study[1]. We speculated that a differential effect of HLA-B27 on cytokine production (TNF-α and IFN-γ), if true, may be demonstrable also among patients with AS. Indeed, we observed a trend for a lower percentage of TNF-α and IFN-γ-producing T cells among HLA-B27-positive patients with AS compared to HLA-B27-negative patients from the GESPIC, yet the differences were statistically insignificant. Despite the lack of statistical significance, the percentages of cytokine-positive cells were remarkably similar in the 2 studies: HLA-B27-positive patients with AS from the GESPIC study (n = 92) reported here showed a median percentage of TNF-α+ CD4+ T cells of 7.57%, while in the previous study[1] this number was 5.11% among B27-positive patients with AS (n = 25) and 7.48% among HLA-B27-positive healthy controls (n = 18). In comparison, among HLA-B27-negative subjects, the respective percentages were 10.43% in this study (n = 15 HLA-B27-negative patients with AS) and 9.5% in the previous study on 22 HLA-B27-negative healthy subjects. Thus, in addition to the previous study, where there were no HLA-B27-negative patients with AS, the data from this study suggest a difference in cytokine production also between HLA-B27-positive and HLA-B27-negative patients with AS. Whether this difference has an effect on AS susceptibility cannot be determined by our study, but may be the case. Since the percentage of TNF-α+ CD4+ T cells was of similar magnitude among HLA-B27-negative patients with AS in this study and among HLA-B27-negative healthy controls in the previous study, we can only speculate that in HLA-B27-negative patients with AS, factors other than TNF-α production by CD4 T cells are likely to operate as susceptibility factors. Unfortunately, we could not investigate again in healthy HLA-B27-positive and negative controls in this study because there were no healthy controls in GESPIC.

The TNF-α gene is located on the short arm of chromosome 6 within the HLA class III region, ~250 kilobases centromeric of the HLA-B locus and 850 kilobases telomeric of HLA-DR. Several polymorphic areas were identified within the TNF gene locus, including –238 and –308 G/A promoter polymorphisms. The data about the influence of the TNF-α promoter polymorphism on TNF-α expression are conflicting. Several studies showed a significant association between the presence of the alternative allele A at position –308 and higher TNF-α production[14,23,24,25,26,27,28], while others did not find such an association, or reported an even lower TNF-α production in the presence of the A allele[29,30,31,32]. The same situation was observed for the –238 promoter polymorphism: some authors found lower production of TNF-α in the presence of the alternative rare (A) allele[31,33], while in other studies there was a lack of such association[29,32,34]. Notably, the influence of TNF-α promoter polymorphisms had been investigated using different cell cultures, different stimuli, and different assays that might explain some of the divergent results.

The presence of the A allele at TNF–308 was associated with significantly lower percentages of TNF-α-producing CD4+ and CD8+ cells in patients with AS. This is in con-

trast to our previous report[1], in which we found a higher percentage among the 6 HLA-B27-positive individuals carrying an A allele at –308 (2 patients with AS and 4 healthy controls). Further, we found a lower percentage of IL-10-producing CD8+ T cells in B27-positive patients with AS as compared to B27-negative patients in this study, a finding that is again contrary to the result from the previous study[1]. The reasons for these differences between the 2 studies are not entirely clear but factors such as a relatively small sample size in the 2 studies may play a role.

Despite a substantially larger number of patients in our current study, it was still underpowered in some aspects: this study had a 61% power to detect a difference for TNF-α+ CD4+ cells and a power of 33% for a difference of TNF-α+ CD8+ T cells if the findings of the first study were true. It is important to mention that adequate power and sample size calculation is difficult to perform for such an analysis because of the large number of measurements to be analyzed (in fact every measurement needs a separate power calculation), non-normal data distribution, and unequal sample size (e.g., the power to detect a difference in the percentage of CD8+ TNF-α-producing cells between HLA-B27-positive and negative subjects was 75%, but only 33% for the difference between –308 GG and GA carriers). This could be a reason for several nonsignificant trends that we found. Increasing the sample size, and particularly increasing the proportion of HLA-B27-negative patients with AS, would increase power and potentially provide clearer results. Unfortunately, in this analysis we could not include more patients with AS from the GESPIC because all patients for whom DNA material and frozen T cells were available had been included. Nevertheless, the trend for a difference of T cell production of TNF-α, IFN-γ, and IL-10 between HLA-B27-positive and HLA-B27-negative patients with AS in the entire cohort (and a statistically significant difference for TNF-α-producing CD8+ T cells after exclusion of patients treated with anti-TNF agents), which is similar to the findings of the previous study[1], together with the similar proportion of cytokine-positive CD4+ and CD8+ T cells in both studies, suggest that the observed effects of HLA-B27 on cytokine production may be real, and may play a role in AS pathogenesis.

In these patients with AS from the GESPIC we had the chance to investigate the relation of genetic and functional cytokine data with clinical measurements. We did not find any association or correlation of the percentage of cytokine-producing T cells or the presence of alternative alleles at TNF–238 and –308 with clinical manifestations in AS, disease activity, functional status, mobility measures, radiographic scores, or levels of acute-phase reactants.

Our study provides confirmatory data for a decreased TNF-α, IFN-γ, and IL-10 production by T cells mediated by HLA-B27, a process that may play a role in AS disease development in HLA-B27-positive subjects. No conclusive data related to the –308 TNF gene promoter polymorphisms' influence on cytokine production in AS were obtained, most likely because of lack of power. Even greater sample sizes would be required to study any effect of the TNF–238 polymorphism because of the rareness of alternative allele carriers. Major effects were not observed on clinical manifestations in AS mediated by the TNF–308 gene polymorphisms or mediated by the percentage of cytokine-producing peripheral T cells.

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