EXTENDED REPORT

Distinct tumour necrosis factor α , interferon γ , interleukin 10, and cytotoxic T cell antigen 4 gene polymorphisms in disease occurrence and end stage renal disease in Wegener's granulomatosis

B M Spriewald, O Witzke, R Wassmuth, R R Wenzel, M-L Arnold, T Philipp, J R Kalden

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See end of article for authors' affiliations

Correspondence to:
Dr Bernd M Spriewald,
Institute for Clinical
Immunology, Department
of Medicine III,
Glückstrasse 4a, 91054
Erlangen, Germany;
bernd.spriewald@med3.
imed.uni-erlangen.de

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Background: Cytokines and T cell regulatory proteins play an important role in the pathogenesis of Wegener's granulomatosis (WG).

Objective: To investigate cytokine and cytotoxic T cell antigen-4 (CTLA4) gene polymorphisms and HLA class II alleles in generalised WG.

Methods: The distribution of cytokine and cytotoxic T cell antigen 4 (CTLA4) gene polymorphisms and HLA class II alleles was analysed in 32 patients with generalised WG and 91 healthy controls. Genotyping was carried out for HLA-DRB1 and HLA-DQB1 and for polymorphism of the genes encoding TNF α (–238, –308, –376), TGF β (codon 10 and 25), IFN γ (+874), IL6 (–174), IL10 (–592, –819, –1082), CTLA4 (–318, +49), and the (AT) $_n$ repeats of the CTLA4 gene. In addition, stratification analysis was carried out according to the presence (n = 15) or absence (n = 17) of end stage renal disease.

Results: An increase in the IFN γ +874 T/T (odds ratio (OR) = 3.14) and TNF α –238 G/A (OR = 5.01) genotypes was found in WG patients. When ESRD positive and negative patients were compared, the IFN γ +874 A/A and the CTLA4 –318 C/C genotypes were found more often in the ESRD subgroup (OR = 10.6 and OR = 2.25). WG patients without ESRD had a higher frequency of the IL10 GCC/ACC promoter genotype (OR = 0.13) and long CTLA4 (AT) $_n$ repeats (OR = 0.4). No effect was seen for HLA-DR and –DQ markers.

Conclusions: Disease susceptibility and clinical course in WG may be associated with distinct polymorphisms of cytokine and CTLA4 genes.

Wegener's granulomatosis (WG) is a rare disease characterised by necrotising granulomatous lesions and small vessel vasculitis.¹ In its generalised form the disease mainly affects the respiratory tract and the kidneys. A characteristic feature of WG is the presence of antineutrophil cytoplasmic autoantibodies (ANCA) directed against proteinase 3 (PR3), showing a cytoplasmic staining pattern on immunofluorescence assay. Previous immunogenetic studies have suggested several associations of HLA genes with WG.² However, despite the important role that T cells play in WG, no obvious and consistent HLA association was identified.^{2–3} As disease heterogeneity may hamper the identification of immunogenetic factors in ANCA associated vasculitis,³ we focused our study on PR3-ANCA positive WG patients with generalised disease involving the kidneys.

Activated T cells can be found in granulomatous lesions and in peripheral blood from patients with generalised WG.^{4–7} Furthermore, the immune response in WG seems to be skewed towards a T helper (Th)1-like cytokine secretion pattern in granuloma derived T cells and in peripheral T cells.^{7–9} Proinflammatory cytokines as well as transforming growth factor β (TGF β) have been shown to enhance translocation of PR3, the main target of ANCA autoantibodies in WG, to the cell surface of neutrophil leucocytes and presumably endothelial cells.^{10–12} Binding of ANCA to cell surface PR3 enables activation of neutrophils, which can adhere to endothelial cells following cytokine mediated upregulation of adhesion molecules, promoting vascular and tissue damage (reviewed by Reumaux *et al*¹³ and Harper *et al*¹⁴). Owing to their important role in the pathogenesis of WG, polymorphisms in cytokine and T cell

regulatory genes have become a focus of interest for immunogenetic analyses. Recent studies suggested that both interleukin 10 (IL10)^{15–16} and cytotoxic T cell antigen 4 (CTLA4) polymorphisms are associated with WG.¹⁷ No association was found, however, for the –308 tumour necrosis factor α (TNF α)^{17–18} and TGF β codon 25 polymorphism.¹⁶

In the present exploratory study we analysed HLA-DRB1 and HLA-DQB1 alleles and polymorphisms in the interferon γ (IFN γ) (intron1+874), TNF α (promotor –238, –308, –376), TGF β (codon 10 and 25), IL6 (promotor –174), IL10 (promotor –592, –819, –1082), and CTLA4 (promotor –318, intron1 +49, AT repeats) genes in a well defined cohort of PR3-ANCA positive patients with generalised WG and renal involvement.

METHODS

Patients and controls

Thirty two unrelated white patients with PR3-ANCA positive generalised WG were investigated after giving their informed consent. All patients fulfilled the diagnostic criteria of the American College of Rheumatology¹⁹ and the definitions of the Chapel Hill consensus conference.²⁰ They all had generalised disease with WG related renal involvement, as indicated by raised serum creatinine or biopsy proven morphological changes.

Abbreviations: ANCA, antineutrophil cytoplasmic autoantibodies; ESRD, end stage renal disease; IFN γ , interferon γ ; IL, interleukin; TGF β , transforming growth factor β ; TNF α , tumour necrosis factor α ; WG, Wegener's granulomatosis

The patients were seen and treated at the department of nephrology, University Hospital Essen, Germany, and had received standard immunosuppressive therapy, based on cyclophosphamide and corticosteroids. The genotyping results of the patient cohort were compared with those of 91 healthy white controls. Subsequently, WG patients were stratified on the basis of the presence or absence of end stage renal disease (ESRD) requiring haemodialysis or kidney transplantation. The resulting subgroups were similar with regard to age at diagnosis and disease duration (table 1).

DNA extraction

DNA was extracted from peripheral blood samples using the QIAamp DNA blood kit (Qiagen, Kiel, Germany).

HLA-DRB1 and HLA-DQB1 genotyping

HLA-DRB1 and HLA-DQB1 genotyping was carried out using the ELPHA® low resolution polymerase chain reaction (PCR) single strand oligonucleotide (PCR-SSO) typing kit, according to the manufacturer's instructions (Biotest AG, Dreieich, Germany). This typing scheme allowed the resolution of HLA-DRB1*01 to *10 including the splits of DRB1*02, *05, and *06, as given in table 2. For HLA-DQB1, the most common alleles were detected (table 2). No further differentiation of DQB1*02 and *04 was undertaken.

Cytokine genotyping

The dimorphisms of the cytokine genes encoding TGFβ (codon 10 T/C and 25 C/G), IFNγ (intron1 +874 A/T), IL6 (promoter -174 C/G), and IL10 (promoter -592 C/A, -819 C/T, -1082 G/A) were analysed using a commercial sequence specific primer amplification (PCR-SSP) assay (Cytokine Genotyping Tray, One Lambda, Canoga Park, California, USA). The cytokine phenotype with regard to high, intermediate, or low cytokine expression levels associated with the respective genotype or haplotype was named using published conventions.

TNFα and CTLA4 sequence based typing

The polymorphisms in the promoter region of the TNFα gene (-376 G/A, -308 G/A, -238 G/A) were analysed by direct sequence based typing (PCR-SBT) following amplification with the 5'-CAAACACAGGCCTCAGGACTC-3' forward and 5'-AGGGAGCGTCTGCTGGCTG-3' reverse primer, after 30

Table 1 Stratification of patients with Wegener's granulomatosis defined by end stage renal disease		
Variable	End stage renal disease	
	No (n = 17)	Yes (n = 15)
Age at diagnosis (years) (median (range))	54 (19 to 70)	55 (25 to 62)
Disease duration (years) (median (range))	5 (1 to 10)	6 (1 to 20)
IFNγ +874 T/T (high)	7 (41%)	4 (27%)
IFNγ +874 A/A (low)	1 (6%)	6 (40%)*
TNFα -238 G/A (high)	1 (6%)	5 (33%)
IL10 -1082, -819, -592		
GCC/GCC (high)	1 (6%)	5 (33%)
GCC/ACC (intermediate)	9 (53%)	2 (13%)*
CTLA4 -318 C/C	12 (71%)	15 (100%)+
CTLA4 (AT) _n repeats with n = 25 or more	7 (41%)+	-
*p = 0.033, p _c = 0.066; OR = 10.6 (95% CI, 1.1 to 103), power 65.9%.		
**p = 0.028, p _c = 0.168; OR = 0.13 (95% CI, 0.02 to 0.80), power 70.5%.		
+p = 0.046, p _c = 0.184; OR = 2.25 (95% CI, 1.47 to 3.43), power 15.9%.		
‡p < 0.01, p _c < 0.02; OR = 0.4 (95% CI, 0.24 to 0.64), power 22.7%.		
CI, confidence interval; IFNγ, interferon γ; IL10, interleukin 10; OR, odds ratio; TNFα, tumour necrosis factor α.		

Table 2 Frequency of HLA-DRB1 and HLA-DQB1 alleles in patients with Wegener's granulomatosis and healthy controls

	Patients (n = 32)	Controls (n = 91)
DRB1		
*01	6 (19)	24 (26)
*15	8 (25)	28 (31)
*16	/	3 (3)
*03	9 (28)+	11 (12)
*04	9 (28)	21 (23)
*11	5 (16)	19 (21)
*12	4 (13)	4 (4)
*13	5 (16)	24 (26)
*14	3 (9)	8 (9)
*07	7 (22)	20 (22)
*08	3 (9)	7 (8)
*09	/	1 (1)
*10	1 (3)	2 (2)
DQB1		
*02	10 (31)	29 (32)
*0301	5 (16)+	30 (33)
*0302	6 (19)	18 (20)
*0303	4 (13)	4 (4)
*04	3 (9)	5 (6)
*0501	7 (22)	25 (28)
*0502	/	4 (4)
*0503	2 (6)	6 (7)
*0601	/	2 (2)
*0602	8 (25)	29 (32)
*0603	3 (9)	12 (13)
*0604	1 (3)	9 (10)
*0609	/	2 (2)

Values are n (%).
+p value not significant.

cycles using 62°C as the annealing temperature. Polymorphisms in the promotor and first exon of the CTLA4 gene (-318 C/T, +49 A/G) were determined by PCR-SBT following amplification with 5'-AAGTCTCCACTTAGTTATC CAGA-3' as forward and 5'-GGGATGAAGAGAAGAAAA AAC-3' as reverse primer after 30 cycles, using 55°C as the annealing temperature. The PCR products were directly sequenced for both strands using the ABI BigDye® Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, California, USA) and the initial amplification primers.

To analyse the (AT)_n repeats in the 3' untranslated region of the CTLA4 gene, amplification using a fluorescence labelled antisense primer was carried out with the forward 5'-GCCAGTGATGCTAAAGGTG-3' and reverse (FAM) 5'-GTTTCTTAACATACGTGGCTCTATGCA-3' primer, for 30 cycles with 55°C as annealing temperature. The PCR products were analysed on an ABI 310 using the GeneScan® software and 400 HD (ROX) size standard (Applied Biosystems). To define the number of AT repeats, homozygous samples were directly sequenced as described above using 5'-GTTTA TAGCCGAAATGATCT-3' as forward and 5'-AAAGACACTG CCATATAGTG-3' as reverse primers. The (AT)_n length polymorphism could thus be translated into the number of AT repeats, allowing better comparison between different studies.

Statistical analysis

Phenotype and genotype frequencies were compared between the patient and control group by the Yates corrected χ² test for 2x2 tables or the Fisher's exact test as appropriate. Odds ratios (OR) were calculated with 95% confidence intervals (CI). Fisher's exact test was used for the subgroup analysis of the patient cohort. A probability (p) value of <0.05 was considered significant. SPSS version 10.0 (SPSS Inc, Chicago, Illinois, USA) was used for all calculations. In addition, significant p values were subjected to Bonferroni correction

(pc) by multiplying them by the number of antigens/alleles of a given locus compared for each HLA specificity or allele or single nucleotide polymorphism for which significance was found before correction. Reporting both the p values balances the need for minimising the risk of reporting spurious associations and the chance of overlooking a true association. Study size calculations were carried out for unmatched case-control studies with equal size for a power of 90% and a level of significance of $p = 0.05$ (two sided), using the relative risk and the exposure rate among controls obtained in this study, according to Schlesselman²¹:

$$n = 2\bar{p}\bar{q}(z_\alpha + z_\beta)^2 / (p_1 - p_0)^2$$

Similarly, power calculations were undertaken for significant results based on the study size, exposure rate among controls, and the relative risk seen in this study:

$$\text{Power} = 1 - \beta = P(Z \leq z_\beta);$$

$$z_\beta = [n(p_1 - p_0)^2 / 2\bar{p}\bar{q}]^{1/2} - z_\alpha$$

RESULTS

HLA-DRB1 and HLA-DQB1 analysis

The results of the HLA-DRB1 and HLA-DQB1 genotyping are summarised in table 2. No significant differences in allele frequencies between WG patients and healthy controls were found. There was only a trend towards a higher frequency for DRB1*03 (28% WG v 12% HC, NS) and a slight reduction of DQB1*0301 in WG patients (16% WG v 33% HC, NS).

IFN γ , TNF α , TGF β , IL6, and IL10 genotypes in WG

A higher frequency of the IFN γ intron1+874 T/T genotype was found in WG patients than in controls (34% v 14%, $p = 0.027$, $p_c = 0.054$; OR = 3.14 (95% CI, 1.23 to 8.01), power = 46%). Also, the TNF α -238 G/A promoter genotype was found more often in WG patients (19% v 4%, $p = 0.029$, $p_c = 0.058$; OR 5.01 (95% CI, 1.31 to 19.14), power 40.5%). No associations were found for the other two promoter polymorphisms. No significant differences between the WG patients and controls were seen for the IL6 -174 C/G polymorphism and the TGF β (codon 10 T/C and 25 C/G) and IL10 (promoter -592 C/A, -819 C/T, -1082 G/A) haplotype or genotype frequencies (data not shown). The results are summarised in table 3.

CTLA4 genotypes in WG

No differences in the CTLA4 -318 C/T promoter and the exon1 +49 A/G frequencies were noted between the patient and control group. Upon analysis of the AT repeats in the 3' untranslated region of the CTLA4 gene we found 20 alleles with between seven and 33 AT repeats, giving rise to 400 potential genotypes. The number of identified AT repeats was in accordance with a previous report.²² The genotypes 7/7 and 7/16 represented half or more than half of the 38 genotypes actually identified in both cohorts. No significant differences in the allele or genotype distribution were noted between the two cohorts (data not shown).

Stratification analysis of WG patients

The patient cohort was divided according to whether or not the patients had developed end stage renal disease requiring haemodialysis or kidney transplantation. Both subgroups were similar with regard to age at diagnosis and disease duration (table 1). The IFN γ +874 A/A genotype was more prevalent in the ESRD subgroup (table 1). In contrast, the IFN γ +874 T/T genotype distribution did not show any

Table 3 Cytokine gene polymorphism frequency in patients with Wegener's granulomatosis and controls

Genotype	Phenotype	Patients (n = 32)	Controls (n = 91)
IFNγ intron +874 (A/T)			
T/T	High	11 (34)*	13 (14)
T/A	Intermediate	14 (44)	53 (58)
A/A	Low	7 (22)	25 (28)
TGFβ1 codon 10 (T/C)/25 (C/G)			
T/T G/G	High	6 (19)	21 (23)
T/C G/G	High	13 (41)	44 (48)
T/C G/C	Intermediate	3 (9)	7 (8)
C/C G/G	Intermediate	6 (19)	14 (15)
T/T G/C	Intermediate	1 (3)	/
C/C G/C	Low	2 (6)	4 (4)
C/C C/C	Low	1 (3)	1 (1)
T/T C/C	Low	/	/
T/C C/C	Low	/	/
IL6 promoter -174 (C/G)			
G/G	High	14 (44)	27 (30)
G/C	High	14 (44)	52 (57)
C/C	Low	4 (13)	12 (13)
IL10 promoter -1082 (G/A), -819 (C/T), -592 (C/A)			
GCC/GCC	High	6 (19)	19 (21)
GCC/ACC	Intermediate	11 (34)	27 (30)
GCC/ATA	Intermediate	11 (34)	24 (26)
ACC/ACC	Low	1 (3)	10 (11)
ACC/ATA	Low	3 (9)	9 (10)
ATA/ATA	Low	/	2 (2)
TNFα promoter -376 (G/A), -308 (G/A), -238 (G/A)			
-376 G/G	Intermediate	30 (94)	89 (98)
-376 G/A	Intermediate	2 (6)	2 (2)
-376 A/A	Intermediate	/	/
-308 G/G	Low	24 (75)	79 (87)
-308 G/A	High	6 (19)	10 (11)
-308 A/A	High	2 (6)	2 (2)
-238 G/G	Low	26 (81)	87 (96)
-238 G/A	High	6 (19)†	4 (4)
-238 A/A	High	/	/

* $p = 0.027$, $p_c = 0.054$; OR = 3.14 (95% CI, 1.23 to 8.01), power 46%.

† $p = 0.029$, $p_c = 0.058$; OR = 5.01 (95% CI, 1.31 to 19.14), power 40.5%.

CI, confidence interval; IFN γ , interferon γ ; IL, interleukin; OR, odds ratio; TGF β 1, transforming growth factor β 1; TNF α , tumour necrosis factor α .

heterogeneity. A trend towards a higher frequency in the ESRD subgroup was observed for the TNF α -238 G/A, the IL10 GCC/GCC, and the CTLA4 -318 C/C genotype. In contrast, the IL10 GCC/ACC genotype was found more often in the non-ESRD subgroup. CTLA4 (AT)_n alleles with 25 or more repeats were found in the non-ESRD subgroup only.

Study size calculations

Based on the uncorrected p values, significant associations were subjected to study size calculations aiming at a level of significance $\alpha = 0.05$ (two sided) and power $\beta = 0.1$ (that is, 90% and an equal size of patient and control groups). For the analysis of IFN γ +874 T/T a cohort of at least 97 patients was needed. Similarly, the study of TNF α -238 G/A polymorphism required at least 113 patients. For the comparison of ESRD positive and ESRD negative patients for IFN γ +874 A/A, at least 32 patients each would be required, and for the study of the CTLA4 -318 C/C polymorphism a minimum of 195 patients. The study size requirements were 29 and 122 for the IL10 GCC/ACC promoter polymorphism and for the long CTLA4 (AT)_n repeats, respectively.

DISCUSSION

In this exploratory study, we focused on a cohort of PR3-ANCA positive WG patients with renal involvement, to explore the relevance of HLA markers, cytokine polymorphism, and genetic CTLA4 variants for disease susceptibility and

the occurrence of end stage renal disease as a serious complication of WG.

As WG is a rare disease with an approximate prevalence of 23.7 per million adults,¹ studies such as ours and others recently published^{15–18, 23} on the immunogenetics of WG are prone to deficient statistical power to clarify the associations of genetic markers with disease susceptibility and outcome. Thus the heterogeneity detected in this study requires further investigation in larger cohorts, possibly multicentre studies, as confirmed by the calculations of the sample sizes needed to provide definitive evidence of the modulatory effects of immunoregulatory genes. Nevertheless, our study may help in the selection of immunoregulatory genes for further study in this candidate gene approach, as discussed in more detail below.

In our study we did not observe any predispositional or preventive effects of the HLA-DRB1 and DQB1 alleles analysed. When IFN γ intron 1 polymorphism was investigated, a complex association pattern was seen. While the IFN γ high expressor T/T genotype²⁴ was associated with disease occurrence, it was not increased in the subgroup of WG patients who had developed ESRD. In contrast, the +874 A/A genotype—a low expressor phenotype²³—was increased in ESRD positive patients compared with the remainder. IFN γ as part of a T-helper 1 cytokine pattern has been implicated previously in the pathogenesis of WG.^{7–9} Thus distinct IFN γ polymorphisms may modulate disease susceptibility and outcome.

Similarly, TNF α , another important proinflammatory cytokine produced in peripheral or granuloma T cells from WG patients, seems to play an important role in the pathogenesis.^{7–9} Our data indicate a role for the TNF α –238 G/A genotype, which is associated with a high cytokine expressor phenotype²⁶ for disease susceptibility and renal involvement. The observed lack of association of WG with the TNF α promoter –308 polymorphism is in accordance with previous studies.^{18, 23, 27}

Interleukin 10 seems to play a dual role in both localised and generalised WG.^{4, 7} Moreover, distinct IL10 polymorphisms have previously been associated with WG.^{15, 16} In our cohort we did not find any differences in the frequencies of the IL10 promotor polymorphisms investigated. However, in the subgroup analysis, WG patients without ESRD showed a higher prevalence of the IL10 GCC/ACC promoter haplotype, which had been associated with an intermediate expressor phenotype.^{25, 28} Thus IL10 polymorphisms may be related to disease course in WG. The possibility that cytokine polymorphisms may influence disease outcome in WG is supported by a recent study by Borgmann and colleagues, who showed that the proinflammatory genotype of IL1 and the IL1 receptor antagonist were associated with ESRD rather than with disease susceptibility in patients with PR3-ANCA vasculitis.²⁹

Two previous studies had suggested associations of the CTLA4 (AT)_n repeat and promotor polymorphisms with WG.^{17, 23} However, in our study we did not find a reduction in the CTLA4 –318 C/C genotype, though in the subgroup analysis the –318 C/C genotype was increased in WG patients who did not develop ESRD. We could not detect a reduction in the frequency of the shortest (AT)_n allele and the resulting genotypes, in contrast to Huang *et al.*²³ However, in the subgroup analysis alleles with 25 or more AT repeats were found exclusively in the WG subgroup which did not develop ESRD. These discrepancies between studies may reflect the low power of the individual studies, or differences in patient selection and disease heterogeneity.

Patients with systemic vasculitis including ANCA associated vasculitis have raised plasma levels of TGF β , which can also serve as a translocation factor for proteinase 3.^{11, 30}

Despite this potential role of TGF β in the pathogenesis of WG, we did not observe any distinction between the WG and control group with respect to the TGF β codon 10 and 25 polymorphisms.

Conclusions

The data suggest that distinct IFN γ polymorphisms are associated with disease susceptibility and outcome in generalised WG. In addition, the TNF α –238 G/A genotype may contribute to disease susceptibility. T cell regulatory gene polymorphisms (IL10, CTLA4) appear to be associated with disease outcome alone rather than with susceptibility to generalised WG.

Authors' affiliations

B M Spriewald, M-L Arnold, J R Kalden, Institute for Clinical Immunology, Department of Medicine III, University Erlangen-Nürnberg, Erlangen, Germany

O Witzke, R R Wenzel, T Philipp, Department of Nephrology, University Hospital Essen, Essen, Germany

R Wassmuth, Institute for Transplantation Diagnostics and Cell Therapeutics, University Medical Centre, Düsseldorf, Germany

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