

# Polymorphisms in genes coding for mediators in the interleukin cascade and their effect on susceptibility to sarcoidosis in the Slovenian population

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Received February 9, 2007; Accepted March 23, 2007

**Abstract.** Sarcoidosis is a chronic inflammatory disease characterised by granulomatous inflammation in various organs. As genetic factors have been implicated in its aetiology, in our study we investigated whether the promotor polymorphisms in three genes coding for inflammatory mediators interleukin-6 (IL-6), interleukin-12 (IL-12), and interleukin-18 (IL-18) could be associated with susceptibility to sarcoidosis. The study sample consisted of 104 patients with sarcoidosis and 100 healthy control subjects. Following DNA isolation from peripheral blood the IL-6/-174G>C single nucleotide polymorphism (SNP), the IL-12B/4 bp insertion polymorphism, and the IL-18/-137G>C SNP were characterised by polymerase chain reaction. Our results showed an increased frequency of IL-6/-174C allele ( $p<0.01$ , OR=2.05, 95% CI=1.32-3.16), and also an increased genotype frequency of IL-6/-174 CC and CG carriers ( $p<0.01$ , OR=2.78, 95% CI=1.49-5.19) among sarcoidosis patients in comparison with healthy controls. Allele and genotype frequencies did not differ significantly between cases and controls for either the IL-12B/4 bp insertion polymorphism or for the IL-18/-137G>C polymorphism. In conclusion, we demonstrated that the -174G>C promotor polymorphism in the IL-6 gene may be a risk factor for sarcoidosis.

## Introduction

Sarcoidosis is a chronic multisystem disorder of unknown aetiology, characterised by infiltration of T lymphocytes and macrophages, and granuloma formation in multiple organs and consequential distortion of normal organ architecture (1). Available evidence suggests the pathophysiology of the disorder to be an exaggerated immune response to a variety of antigens and autoantigens. This immune response is characterised by infiltration with mostly CD4<sup>+</sup> T-lymphocytes which spontaneously release inflammatory mediators interleukin-2 (IL-2), interleukin-12 (IL-12) and interferon- $\gamma$  (IFN- $\gamma$ ), and thus promote the development of T<sub>H</sub>1 predominant immune response (2).

As sarcoidosis is primarily a disease characterised by the presence of increased numbers of inflammatory cells, various immune mediators have been examined for their involvement in the pathogenesis of sarcoidosis. Expression and proteomic studies have shown alterations in expression and release of various substances, especially interleukins (3,4). Polymorphisms in genes that encode interleukins could therefore predispose an individual to sarcoidosis.

Interleukin-6 (IL-6) is a pro-inflammatory cytokine which induces activation and proliferation of T cells and contributes to formation of granuloma. Increased concentrations have been found in lung specimens of patients with sarcoidosis (4). A polymorphism which affects its transcriptional activity has been described at position -174, substituting G for C (5).

IL-12 causes differentiation of naïve T cells into T<sub>H</sub>1 cells and induces the production of IFN- $\gamma$  by T<sub>H</sub>1 cells. It is composed of two units (p35 and p40) which together constitute the biologically active form p70. In the IL-12B gene, which encodes the p40 subunit of IL-12 (6), a polymorphism has been described; a microinsertion 3 bp upstream of the transcriptional start site, which in its heterozygous state causes reduced expression of the IL-12 gene and reduced production of the IL-12 p70 subunit (7).

Interleukin-18 (IL-18) is an important IFN- $\gamma$ -inducing factor thereby promoting the T<sub>H</sub>1 response. Substitution of G

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**Key words:** sarcoidosis, polymorphism, interleukin-6, interleukin-12, interleukin-18, association study

with C at position -137 has been described in the promoter region which disrupts the histone H4 gene transcriptional factor-1 binding site in the IL-18 promoter. Homozygotes for the -137G allele variant show enhanced expression of IL-18 mRNA (8).

In our study, PCR analysis of the IL-6/-174G>C, IL-12B/insertion and IL-18/-137G>C polymorphisms was performed in Slovenian patients with sarcoidosis in order to determine whether they contribute to the disease susceptibility.

## Materials and methods

**Subjects.** Subjects diagnosed with sarcoidosis were recruited from an ongoing sarcoidosis registry started in the year 2000 at the University Clinical Centre Ljubljana, Department of Pulmonary and Allergic Diseases. Diagnosis of sarcoidosis was based on clinical assessment, radiographic presentation, bronchoalveolar lavage (BAL) and biopsy specimens from the lung, skin or lymph nodes after other granulomatous diseases were excluded. One hundred and four patients were included in the study: 36 males and 68 females; age range, 21-68 years; and mean age, 41 years. The patients were followed-up 3-10 years after confirmation of diagnosis. Regarding the clinical presentation at diagnosis, pulmonary lymph nodes were affected in 89, and lung interstitium in 73 patients. According to the classification system defined by DeRemee (9), 15 patients were in stage I, 55 in stage II, 16 in stage III and 2 in stage IV at the time of the first visit. Extrapulmonary organ involvement was found in 35 patients. Various types of skin involvement were found in 31 patients and Löfgren's syndrome was present in 19. Eight patients had arthralgias, 10 had extrapulmonary node involvement, and 5 had salivary gland involvement. In 15 patients, involvement of the parenchymal organs such as the liver, spleen, kidney or heart; and in 9 patients, various types of neural involvement were present.

The control groups consisted of 100 age- and sex-matched healthy blood donors. All patients and control subjects were Slovenian, and not related to each other.

All subjects participated in the study after they had given their full informed consent. The study was approved by the state ethics committee.

**Molecular analysis.** Following DNA isolation from blood leukocytes by standard procedure, polymerase chain reaction method was performed to detect the -174G>C polymorphism of the IL-6 gene, the IL-12B/insertion polymorphism and the -137G>C polymorphism in the IL-18 gene.

The IL-6/-174G>C polymorphism was determined by performing PCR and restriction of amplified products. The PCR final reaction mix consisted of 1X PCR buffer, 0.2 mM dNTP, 1.3 mM MgCl<sub>2</sub>, 500 nM of primers, 1 unit of Taq polymerase (Promega, Madison, WI), bidistilled H<sub>2</sub>O and 500 ng of DNA. The primer sequences were: IL-6/-174 (G/C) forward, 5'TGACTTCAGCTTTACTCTTGT3'; and IL-6/-174 (G/C) reverse, 5'CTGATTGGAAACCTTATTAAG3'.

The protocol for PCR amplification was: initial denaturation at 94°C for 10 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 35 sec

and elongation at 72°C for 1 min, and final extension at 72°C for 10 min.

Following the amplification, the 198-bp-large PCR products were digested with 5 units of Hsp92II restriction enzyme and visualized on 3% agarose gel with ethidium bromide. When the G allele was present the restriction enzyme cut the product only at one site, producing two fragments 31 bp and 167 bp long. In the presence of the C allele, the restriction enzyme cut the PCR reaction product at two sites, producing three fragments, 31 bp, 41 bp and 122 bp long.

The IL-12B/insertion polymorphism was analysed according to a previously published method (7). The final reaction mix consisted of 1X PCR buffer, 0.2 mM dNTP, 2.5 mM MgCl<sub>2</sub>, a 300-nM concentration of each primer, 0.3 units of Taq polymerase (Promega), bidistilled H<sub>2</sub>O and 500 ng of DNA. The primer sequences were: IL-12 forward, 5'TGGATTGTGAAGTGGGACAT3'; and IL-12 reverse, 5'TAATGTGGTCATTGGCAGGT3'.

The protocol for PCR amplification was as follows: initial denaturation at 94°C for 3 min, then 35 cycles of denaturation at 95°C for 20 sec, annealing at 55°C for 20 sec and elongation at 72°C for 30 sec, and final extension at 72°C for 10 min.

Following amplification, the IL-12B/insertion polymorphism was visualized on Spreadex® EL600 mini gel (Elchrom Scientific AG, Switzerland) after running it for 83 min at 120 V and at a temperature of 55°C. The polymorphism was determined by the presence of either the 162-bp (insertion) or 158-bp (no insertion) allele.

The IL-18/-137G>C polymorphism was analyzed according to sequence-specific PCR (SSP-PCR) protocol as previously described (8). The final reaction mix consisted of 1X PCR buffer, 0.2 mM dNTP, 2.5 mM MgCl<sub>2</sub>, a 300-nM concentration of each primer, 0.5 units of Taq polymerase (Promega), bidistilled H<sub>2</sub>O and 100 ng of DNA. The primer sequences were: IL-18/-137 control primer, 5'CTTTGC TATCATTCCAGGAA3'; IL-18/-137G>C G-specific forward primer, 5'GTTGCAGAAAGTGTAATAAATT ATTAC3'; IL-18/-137G>C T-specific forward primer, 5'GTTGCAGAAAGTGTAATAAATTATTAA3'; and IL-18/-137 reverse, 5'TAACCTCATTCAGGACTTCC3'.

The protocol for PCR amplification was as follows: initial denaturation at 94°C for 2 min, then 5 cycles of denaturation at 94°C for 20 sec followed by 68°C for 60 sec and 25 cycles of 94°C for 20 sec, 62°C for 20 sec, 72°C for 40 sec and final extension at 72°C for 2 min.

After the amplification, the PCR products were visualized on 2% agarose gel with ethidium bromide.

**Statistical analyses.** The significance of association was analyzed using the Chi-square test ( $\chi^2$ ). Odds ratios (OR) and their respective 95% confidence intervals (CI) were also calculated to compare allelic and genotype distribution in patients and control subjects. Analyses were performed using the SPSS package (SPSS 13.0, SPSS Inc., Chicago, IL).  $\chi^2$  tests for deviation from Hardy-Weinberg equilibrium were calculated. Tests of statistical power were performed using PS Power and Sample Size Calculations program, version 2.1.30, available at <http://www.mc.vanderbilt.edu/prevmed/>



## Allele and genotype frequencies of the IL-6/-174G&gt;C polymorphism in sarcoidosis patients and controls.

	Sarcoidosis patients n (%)	Controls n (%)	OR (95% CI)	p
Allele				
G	108 (51.9)	106 (68.8)	2.04 (1.32-3.16)	0.001
C	100 (48.1)	48 (31.2)		
Total	208 (100)	154 (100)		
Genotype				
GG	27 (26.0)	38 (49.4)	CC + CG vs. GG 2.78 (1.49-5.19)	0.001
GC	54 (51.9)	30 (40.0)		
CC	23 (22.1)	9 (11.7)		
Total	104 (100)	77 (100)		

ps/index.htm (10). Associations were regarded as significant when they reached  $p \leq 0.05$ .

### Results

An increased frequency of the IL-6/-174C allele was found in patients with sarcoidosis in comparison to the control group ( $p < 0.01$ , OR=2.05, 95% CI=1.32-3.16). Additionally, an increased frequency of IL-6/-174C allele carriers (CC homozygous and CG heterozygous vs. GG homozygous) was found among patients with sarcoidosis ( $p < 0.01$ , OR=2.78, 95% CI=1.49-5.19). Frequencies of alleles and genotypes in the patient and control groups are shown in Table I.

There was no difference in allele and genotype frequencies between the patient and control group for the polymorphisms in the IL-12B and IL-18 genes (Table II).

The calculation of statistical power revealed that in the IL-6 polymorphism analysis, based on 104 patients and 77 genotypes obtained from the control subjects, the power of 0.80 should be achieved to detect a 2.4-fold increase in the frequency of risk genotypes, assuming the percentage of risk genotype in control subjects to be 0.52. In the IL-12B polymorphism analysis with 104 patients and 100 control subjects, the power of 0.80 should be achieved to detect a 2.8 OR, assuming the percentage of risk genotypes in the control subjects to be 0.73. In the IL-18 polymorphism analysis with

Table II. Allele and genotype frequencies of the IL-12B/insertion polymorphism (allele 1, insertion; allele 2, no insertion variant), and of the IL-18/-137G>C polymorphism in sarcoidosis patients and controls.

		Sarcoidosis patients n (%)	Controls n (%)	OR (95% CI)	p
IL-12B	Allele				
	1	105 (50.5)	106 (53.0)	1.11 (0.75-1.63)	0.611
	2	103 (49.5)	94 (47.0)		
	Total	208 (100)	200 (100)		
	Genotype				
	11	24 (23.1)	27 (27.0)	12 + 22 vs. 11 1.23 (0.65-2.33)	0.518
	12	57 (54.8)	52 (52.0)		
	22	23 (22.1)	21 (21.0)		
	Total	104 (100)	100 (100)		
IL-18	Allele				
	G	146 (74.5)	143 (71.5)	1.16 (0.75-1.82)	0.503
	C	50 (25.5)	57 (28.5)		
	Total	196 (100)	200 (100)		
	Genotype				
	GG	55 (56.1)	51 (51.0)	GG vs. GC + CC 1.22 (0.70-2.15)	0.470
	GC	36 (36.8)	41 (41.0)		
	CC	7 (7.1)	8 (8.0)		
	Total	98 (100)	100 (100)		

98 patient genotypes and 100 control subject genotypes, the power of 0.80 should be achieved to detect a 2.3-fold increase in the frequency of risk genotype, assuming the percentage of risk genotype in the control subjects to be 0.49 ( $p < 0.05$ ).

There was no statistical deviation from Hardy-Weinberg equilibrium in all three analyses ( $p < 0.05$ ).

## Discussion

In our study an increased frequency of the C allele at the IL-6/-174G>C promotor polymorphism ( $p < 0.01$ , OR=2.05, 95% CI=1.32-3.16) was demonstrated, as well as an increased frequency of combined GC heterozygotes and CC homozygotes vs. GG homozygotes, at the same polymorphism ( $p < 0.01$ , OR=2.78, 95% CI=1.49-5.19) in patients with sarcoidosis in comparison with the control group. Analysis of allele and genotype frequencies for the IL-12B/insertion polymorphism and the IL-18/-137G>C polymorphism showed no significant differences between the patient and control groups.

IL-6, IL-12 and IL-18 have previously been related to sarcoidosis for their pro-inflammatory effects, and also, few studies have considered the role of polymorphisms in these genes in the susceptibility to sarcoidosis (3,11-19).

Interleukin-6 plays an important role in the regulation of immune response through B and T cell activation, production of acute-phase reactants and production of immunoglobulins (20-22). In sarcoidosis, augmented expression of the IL-6 gene (23) significantly increased spontaneous release of IL-6 by cells in BAL and by peripheral blood mononuclear cells (24-26), and increased levels of IL-6 in BAL fluid, cultured monocytes, alveolar macrophages and alveolar fibroblasts (27-30) have been demonstrated. It has been reported that IL-6 induces T cells to express the IL-2 receptor on their surface which activates and induces the proliferation of T cells in the presence of IL-2 (21). Consequently, IL-6 could affect the susceptibility to sarcoidosis or its clinical presentation/severity, enhancing the positive loop which is caused by spontaneous release of IL-2 from T cells in sarcoidosis.

A -174G>C single nucleotide polymorphism (SNP) in the IL-6 gene promotor region has been identified and related to Alzheimer's disease, atherosclerosis, cardiovascular disease, cancer, non-insulin-dependent diabetes mellitus, osteoporosis, sepsis, systemic-onset juvenile chronic arthritis and other diseases (31). Previous studies have shown that both the -174G and -174C variant can act as risk factors in different diseases (32-38).

The G>C nucleotide change at position -174 has been reported to alter the expression of the IL-6 gene, although it is uncertain which variant actually increases levels of IL-6 (5). Functional studies show that substitution of G with C at the -174 position creates a recognition site for NF- $\kappa$ B transcription factor, which acts as a repressor in HeLa cells (5,39). It has also been demonstrated by luciferase reporter gene assay, that the presence of the -174C allele causes a decreased rate of expression (40).

As sarcoidosis is characterised by increased levels of IL-6 in lung specimens, our finding that -174C could be a predisposing factor may seem in contrast to the results of afore-

mentioned functional studies of the polymorphism. However, the results from investigations on the influence of -174G>C on IL-6 plasma levels in other diseases, generally have indicated that the -174G variant causes increased serum levels of IL-6 (5,31,41,42), although some reports have stated that the -174C variant is associated with increased IL-6 plasma concentration (37,43-45), and others have shown that -174G>C has no influence on IL-6 levels (46). These contradictory results could be due to differences in transcriptional regulation in various tissues, complexity of influences that modulate IL-6 expression, incomplete representation of IL-6 regulatory regions in constructs used in reporter gene experiments and insufficient data on kinetics of IL-6 release in acute and chronic inflammatory stimulation (40,47). In addition, as sarcoidosis is characterised by compartmentalised inflammatory process, measured interleukin-6 levels may not reflect actual processes in the affected organ. In this view, the presence of the IL-6/-174C allele could cause increased levels of IL-6 in the case of sarcoidosis. Results obtained by Grutters *et al.* (34), showing that the -174C variant predisposes to a less favourable outcome characterised by fibrosis, are in agreement with this possibility, as increased IL-6 levels have been related to enhanced fibrosis (48-50).

On the other hand, IL-6 has notable anti-inflammatory effects (51-53). Using knock-out mouse models, a role of IL-6 in dampening the immune response by decreasing the expression of pro-inflammatory cytokine genes and up-regulating anti-inflammatory mediators, has been observed. IL-6 can inhibit production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). It stimulates production of anti-inflammatory mediators (IL-1 receptor agonist and interleukin-10) and increases the release of soluble TNF- $\alpha$  receptors that inhibit TNF- $\alpha$ -mediated pro-inflammatory actions. This shows that the -174C variant, which reduces IL-6 promotor efficiency *in vitro*, could predispose to sarcoidosis by causing a relative deficiency of anti-inflammatory effects of IL-6. Carriers of the -174C polymorphism may not be able to produce as much IL-6 as the -174G polymorphism carriers, and the paucity of IL-6 anti-inflammatory effects would then contribute to the initiation of sarcoid inflammation. Further analyses of the functional significance of -174 SNP in the regulation of IL-6 release and its levels are needed to clarify which allelic variant actually causes increased IL-6 levels in sarcoidosis.

Interleukin-12 and interleukin-18 are cytokines of the T<sub>H</sub>1 type that act in synergy to regulate the activation of T cells. These two cytokines have previously been studied for their role in the pathogenesis of sarcoidosis, and it has been shown that their levels are increased in samples from patients with sarcoidosis (19,54). Both cytokines stimulate T cells to secrete IFN- $\gamma$ , which promotes T cell activation, and IL-12 additionally stimulates differentiation of naïve T cells into T<sub>H</sub>1-cytokine-producing T cells (19). Dysregulation of their effects could therefore cause immunologic alterations presenting exaggerated T cell response of the T<sub>H</sub>1 type which is observed in sarcoidosis. Both genes are polymorphic and contain functional polymorphisms that may affect their expression or levels of their protein product (7,8).

The gene coding for IL-12 contains a functional polymorphism which is a microinsertion of 4 base pairs in the promotor region near the gene transcriptional start site.





SPANDIDOS with heterozygous genotype at this polymorphism exhibited reduced expression of the IL-12 gene and reduced production of the IL-12 p70 subunit in their samples (7). The significance of this polymorphism has not yet been assessed in patients with sarcoidosis. In our study, we have failed to demonstrate its association with susceptibility to sarcoidosis.

Polymorphisms in the IL-18 gene have been analysed for their functionality but Giedraitis *et al* have been unable to obtain conclusive evidence for their functionality. Increased activity of the gene promoters with certain variants of the -137G>C and -607C>A polymorphisms has been demonstrated but these findings have failed to reach statistical significance (8). These potentially functional polymorphisms in the IL-18 gene promoter region were previously studied for association with sarcoidosis in four association studies that gave conflicting results (55). Takada *et al* and Kelly *et al* have shown that the -607C allele of the -607C>A promoter polymorphism in the IL-18 gene was associated with increased susceptibility to sarcoidosis (13,56), whereas Zhou *et al* and Janssen *et al* were unable to confirm this association (14,15). Three of these groups also investigated the significance of the -137G>C polymorphism in the IL-18 gene promoter, but they all failed to show significant association (14,15,56), in accordance with our results.

Our results indicate that carriers of the -174C allele have a 178% increased risk of developing sarcoidosis. Although our results show significant association of the IL-6 promoter/-174C variant with increased susceptibility to sarcoidosis, our small study sample limits its significance. Assessment of larger study samples is needed to further confirm this hypothesis. It is also important to consider that ethnic background can substantially alter allele and genotype frequencies and their effect on disease susceptibility. Before our results can be applied to other ethnic groups, this variation must also be considered and tested through further research. Functional studies of IL-6/-174G>C influence on IL-6 expression, release, and its levels are also needed to explain its role in pathological processes that lead to the development of sarcoidosis.

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