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Study of Toll-like receptor gene loci in sarcoidosis

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Summary

Sarcoidosis is a multi-factorial systemic disease of granulomatous inflammation. Current concepts of the aetiology include interactions of unknown environmental triggers with an inherited susceptibility. Toll-like receptors (TLRs) are main components of innate immunity and therefore TLR genes are candidate susceptibility genes in sarcoidosis. Ten members of the human TLR gene family have been identified and mapped to seven chromosomal segments. The aim of this study was to investigate all known TLR gene loci for genetic linkage with sarcoidosis and to follow positive signals with different methods. We analysed linkage of TLR gene loci to sarcoidosis by use of closely flanking microsatellite markers in 83 families with 180 affected siblings. We found significant linkage between sarcoidosis and markers of the *TLR4* gene locus on chromosome 9q (non-parametric linkage score 2·63, $P = 0{\cdot}0043$). No linkage was found for the remaining TLR gene loci. We subsequently genotyped 1203 sarcoidosis patients from 997 families, 1084 relatives and 537 control subjects for four single nucleotide polymorphisms of *TLR4,* including Asp299Gly and Thr399Ile. This genotype data set was studied by case–control comparisons and transmission disequilibrium tests, but showed no significant results. In summary, *TLR4* – with significant genetic linkage results – appears to be the most promising member of the TLR gene family for further investigation in sarcoidosis. However, our results do not confirm the *TLR4* polymorphisms Asp299Gly and Thr399Ile as susceptibility markers. Our results rather point to another as yet unidentified variant within or close to *TLR4* that might confer susceptibility to sarcoidosis.

Keywords: genetic linkage analysis, sarcoidosis, *TLR4*, Toll-like receptors

Introduction

Sarcoidosis is a multi-organ inflammatory disease with exaggerated cellular immune activity that leads to formation of non-caseating granulomas in affected organs. The aetiology of sarcoidosis is unknown and presumably complex. The lung and adjacent lymph nodes appear to be the first and primary side of immune reaction in the majority of patients [1,2]. This has led to the hypothesis that an inhaled 'sarcoidosis antigen' triggers the process, and a wide variety of organic and inorganic agents have been suspected to cause sarcoidosis. Major candidates are Gram-negative microbes such as mycobacteria and propriobacteria [3,4], but as different substances such as photocopier dust or crystalline silica have also been discussed [5,6]. An inherited susceptibility is assumed to contribute to the risk of sarcoidosis. This risk varies with ethnicity and it is increased greatly in close relatives of patients [7].

Hypothetical candidate susceptibility genes in sarcoidosis presumably play a role in the concerted molecular and cellular interactions of antigen recognition and immune response. Among the first molecules potentially involved in this process are the Toll-like receptors (TLRs), a family of related transmembrane molecules that are expressed on the surface of a variety of antigen-detecting cells. Different TLRs are activated specifically by different pathogen-associated molecular patterns, such as TLR-4 by cell membrane lipopolysaccharides (LPS) of Gram-negative bacteria, TLR-5 by flagillin and TLR-9 by unmethylated cytosine–phosphate–guanosine (CpG)-rich DNA [8]. The downstream signalling pathway includes activation of nuclear factor (NF)-kappa B that has been demonstrated in the

pathogenesis of sarcoidosis [9]. Two of the TLR genes, *TLR4* and *TLR9*, reside at chromosomal positions that have shown linkage peaks in a previous whole genome scan for predisposing gene loci [10]. Taken together, the TLR gene family appears to be a promising target in the search for sarcoidosis susceptibility genes. *TLR2* and *TLR4* gene variations have been studied in the search for susceptibility to sarcoidosis and showed conflicting results [11–14].

The human TLR genes, *TLR1–TLR10*, have been mapped to seven different positions on human chromosomes. *TLR1*, *TLR6* and *TLR10* are clustered at one location on the short arm of chromosome 4, and *TLR7* and *TLR8* on the X chromosome (http://www.ncbi.nlm.nih.gov/Omim). We have studied these seven chromosomal loci and their flanking regions with highly polymorphic DNA markers, so-called microsatellites, for linkage with sarcoidosis in a panel of 83 families with two or more affected siblings. Basically, above-average sharing of microsatellite alleles in the set of affected siblings was an indicator of linkage between the chromosomal locus under study and the risk of sarcoidosis. This approach allows for effective scanning of candidate susceptibility gene regions prior to fine-mapping efforts and the search for disease-associated gene mutations.

Subjects and methods

The study population consisted of two sets of participants: (i) 83 families with two or more siblings with sarcoidosis investigated for the linkage study ('sib-pair families') and (ii) an extended collection of 1203 sarcoidosis patients from 997 families, 1084 relatives and 537 controls for the population- and family-based association experiments. In the linkage study sample, 63 of the sib-pair families have been studied previously with a set of more widely spaced markers in a whole genome linkage experiment [10]. Parents of the sib-pair families were included whenever possible. In families with only one or no parents available, unaffected siblings were included to facilitate parental genotype reconstruction. Altogether, the sib-pair families comprised 302 individuals, including 180 affected siblings, 104 females and 76 males. The average age at diagnosis was 36·7 years [standard deviation (s.d.) 10·9 years] in these patients.

Most of the patients and families were recruited by calls for participation forwarded by the German patients' organization Deutsche Sarkoidose-Vereinigung or by specialized hospitals. All patients were interviewed by telephone or questionnaire, and their doctors were contacted to confirm the diagnosis and to provide records. The diagnosis was confirmed by biopsy in 120 of 180 patients from the sib-pair families, and the clinical course of the disease in combination with radiology and laboratory findings was consistent with the diagnosis of sarcoidosis in the remaining patients.

Seventy of 180 patients from the sib-pair families had acute sarcoidosis, with sudden complaints and recovery within 2 years. Eighty-three patients suffered from chronic sarcoidosis, mainly with a stealthy beginning, and with enduring disease activity for 2 years or longer. Ten patients had acute sarcoidosis in the beginning but experienced a relapse more than 2 years later. The remaining 17 patients showed other phenotypes, e.g. sole cutaneous sarcoidosis, or were detected incidentally by radiography for other reasons and had no specific complaints.

The sib-pair families were genotyped for 28 highly polymorphic microsatellite markers that flanked the chromosomal loci of the TLR genes. Markers were chosen from either a commercially available genome-wide linkage marker set (Research Genetics Inc., Huntsville, AL, USA) or from the public National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/mapview). Details of the TLR loci and marker positions are listed in Table 1. Marker and TLR gene positions are given in Megabase (Mb) on the chromosomal sequence. Recombination fractions used in linkage analysis were derived from genetic linkage map positions (in centiMorgan, cM) whenever available. If no genetic map position was available or if two markers had the same genetic map position, recombination fractions were derived from the distance on the DNA sequence. Nine of the 28 markers (D1S549; D3S2409; D4S408; D4S1625; D4S1627; D4S1629; D4S2632; D9S934; DXS9902) have been studied in 63 families of the present sib-pair families sample on the occasion of a previous whole genome linkage analysis [10]. Microsatellites of the previous whole genome set have been included into the present study to estimate the effect contributed by the additional sib-pair families. Genomic DNA of the participants was prepared and genotypes were determined as described previously [10]. In brief, microsatellite alleles were amplified through a polymerase chain reaction (PCR) amplification technique with M13 labelled primers, and fluorescent PCR products were separated according to size on an automated sequence analysis apparatus [16].

As an internal control, segregation of microsatellite and Single nucleotide polymorphism (SNP) alleles were checked for Mendelian inheritance by use of Pedcheck [17]. GENEHUNTER version 2·0 [15] was used to calculate non-parametric linkage (NPL) probabilities and to perform transmission disequilibrium tests (TDTs) in the sib-pair families.

The sib-pair families are included in a much larger sarcoidosis study population that has been compiled for high throughput genotyping procedures and family-based statistical procedures. The entire collection comprises 1203 patients and 1084 relatives from 997 families. The complete composition of this population has been described previously [18] and is shown in detail in Table 2. All patients completed a questionnaire on the course of disease, and patients from families with more than one patient were also interviewed by telephone. Patients' physicians were contacted to confirm the diagnosis and to provide records. The average age at diagnosis was 36·4 years (s.d. 10·9 years) and

Table 1. Characteristics and non-parametric linkage (NPL) analysis results of 28 microsatellite markers that flank seven Toll-like receptor (TLR) gene loci.

TLR gene	Marker/locus	Cytogenetic position	Sequence position (Mb)	Number of observed alleles	NPL score	*P*-value
TLR1, 6, 10	D4S2632*		35·4	14	0·67	0·25
	D4S1581		37·8	7	0·16	0·43
	TLR1, 6, 10	4p14	38·5			
	D4S2382		39·7	5	−0·26	0·60
	D4S1627*		43·9	9	−1·33	0·91
TLR2	D4S1625*		143·7	8	0·93	0·18
	D4S2934		154·3	10	0·77	0·22
	TLR2	4q32	154·8			
	D4S2999		155·0	11	0·76	0·22
	D4S1629*		158·6	5	0·97	0·17
TLR3	D4S408*		185·4	8	0·75	0·23
	D4S1540		186·5	9	1·44	0·08
	TLR3	4q35	187·2			
	D4S2390		190·0	8	0·71	0·24
	D4S1523		190·7	16	1·02	0·15
TLR4	D9S1776		117·0	10	2·52	0·0060
	D9S1802		118·7	9	2·32	0·0102
	TLR4	9q33	119·5			
	D9S1864		119·5	14	2·63	0·0043
	D9S934*		121·1	8	2·41	0·0081
TLR5	D1S549*		217·7	8	0·26	0·39
	D1S2689		219·7	8	−0·11	0·54
	TLR5	1q41	221·4			
	D1S3462		230·0	8	−0·80	0·79
	D1S321		239·9	8	−0·03	0·51
TLR7, 8	DXS9895		7·4	6	−0·94	0·83
	DXS7104		12·1	13	−0·15	0·56
	TLR7, 8	Xp22	12·8			
	DXS1224		13·1	8	0·29	0·61
	DXS9902*		15·2	7	0·58	0·72
TLR9	D3S2409*		49·4	9	1·01	0·15
	D3S1573		51·1	8	0·98	0·16
	D3S3026		51·9	7	0·89	0·18
	TLR9	3p21	52·2			
	D3S1076		54·1	8	0·74	0·23

Position according to NCBI Map Viewer, build 36·2. Linkage analysis was performed with GENEHUNTER version 2·0 [15]. Nine of 28 microsatellites (marked by asterisks) have been used in a previous whole genome linkage scan [10].

Table 2. Details of the study population of sarcoidosis patients (acute sarcoidosis: sudden complaints and recovery within 2 years; Löfgren's syndrome: acute sarcoidosis with bilateral hilar lymphadenopathy, erythema nodosum and arthropathy; chronic sarcoidosis: stealthy beginning, enduring disease activity for 2 years or longer).

Family structure	Number of families	Number of patients	Male patients	Female patients	Löfgren's syndrome	Acute sarcoidosis*	Chronic sarcoidosis	Other form of sarcoidosis
Single patient, without parents	309	309	108	201	36	83	205	21
Single patient, with one parent	124	124	60	64	10	28	91	5
Single patient, with both parents	385	385	159	226	55	104	257	24
Two patients, siblings	75	150	63	87	28	62	68	20
Two patients, parent and offspring	57	114	47	67	18	46	54	14
Two patients, 2nd- or 3rd-degree relatives	24	48	28	20	6	17	25	6
More than two patients	23	73	38	35	12	30	39	4
Total	997	1203	503	700	165	370	739	94

*Including Löfgren's syndrome.

ranged from 12 to 80 years. Sarcoidosis was confirmed by biopsy in 1001 of 1203 (83·2%) cases. In the remaining 202 patients the clinical course, in combination with radiology and laboratory findings, was consistent with the diagnosis of sarcoidosis.

Further genotyping of the entire collection of DNA was performed at the only locus with significant linkage results, *TLR4*. One intragenic SNP c.-4036A > G (rs2737191) located upstream of the *TLR4* transcription initiation site, and one intronic SNP c.94-837G > A (rs1927911) were genotyped according to the protocol of the supplier of the assays (Assays-on-Demand from Applied Biosystems; http://www.appliedbiosystems.com).

Two downstream coding polymorphisms Asp299Gly (rs4986790) and Thr399Ile (rs4986791) [19,20] were genotyped by use of the *Taq*Man™ technique as described previously [18]. Primer and probes of the Asp299Gly assay were: forward primer 5′-TGACCATTGAAGAATTCCGATT AGCA; reverse primer 5′-ACACTCACCAGGGAAAATG AAGAA; VIC probe 5′-TACCTCGATGATATTATT; FAM probe 5′-CCTCGATGGTATTATT. The Thr399Ile assay contained the following: forward primer 5′-ACCAAGCC TTGAGTTTCTAGATCTCA; reverse primer 5′-GGTAAT AACACCATTGAAGCTCAGATC; VIC probe 5′-TGATTTT GGGACA ATCA; FAM probe 5′-TGATTTTGGGACAACCA. Genotypes of SNPs were checked for conformance with Hardy–Weinberg equilibrium. Allele and genotype frequencies in cases and controls were compared using Pearson's χ^2 test with 1 and 2 degrees of freedom respectively. In addition, TDTs for single markers and four-marker haplotypes were performed in sarcoidosis families and trios (single patients with parents) by using GENEHUNTER version 2·1 [15].

For case–control comparisons of SNP allele and genotype frequencies, genotype information was extracted from the entire collection. One patient per family carrying the phenotype under scrutiny, i.e. Löfgren's syndrome (characterized by acute sarcoidosis with bilateral hilar lymphadenopathy, erythema nodosum and arthropathy), acute sarcoidosis including Löfgren's syndrome, chronic sarcoidosis, and either acute or chronic sarcoidosis, was selected randomly from the sample of sarcoidosis families. This resulted in groups of 152, 323, 680 and 938 unrelated patients with Löfgren's syndrome, acute sarcoidosis (including Löfgren's syndrome) chronic sarcoidosis, and either acute or chronic sarcoidosis respectively. Statistical power of the samples was calculated by use of the program PS (Power and Sample Size Calculations) version 2·1·30 [21].

The control group comprised 537 healthy blood donors recruited through the Department of Transfusion Medicine at Kiel University Hospital, Kiel Germany. The controls consisted of 263 (49%) males and 274 (51%) females with an average age at inclusion of 40·9 years (s.d. 12·8 years). All patients, parents and controls were German by residence. All participants gave written informed consent for participation in the study. Study protocols were approved in writing by the institutional ethics and data protection authorities.

Results

Microsatellite linkage analysis

We have genotyped 302 individuals from 83 families including 180 siblings with sarcoidosis for 28 microsatellite markers from seven chromosomal regions that harbour genes of the TLR gene family. Haplotype construction and linkage analysis was based on 486 meioses in the pedigrees. Haplotype recombinations were observed within families, and their number was consistent with the small number expected from the distance between the markers. For example, the distance between D9S1776 and D9S1802 on the genetic linkage map of chromosome 9 is 2 cM (equivalent to two recombinations in 100 meioses). According to calculations using GENEHUNTER version 2·0, there were 9·4 haplotype recombinations between D9S1776 and D9S1802 to be expected in the sib-pair sample, and 9·2 recombinations were observed. The structure of the sib-pair families allowed for calculation of marker allele and haplotype sharing in 110 sib-pairs. The information content of multi-point analysis at the 14 marker loci directly adjacent to the seven TLR gene loci ranged from 0·68 to 0·84, with an average value of 0·77. We found a significant excess of allele and haplotype sharing, indicating linkage to sarcoidosis at the *TLR4* locus on chromosome 9q, with a maximal non-parametric multi-point linkage score of 2·63 ($P = 0·0043$) for the microsatellite marker D9S1864. This marker is located very close to *TLR4*, at a distance of approximately 10 kb. For the remaining six chromosomal regions under scrutiny, no significant linkage to sarcoidosis was detected in single-point and multi-point analyses. Results of multi-point linkage analysis are compiled in Table 1.

When only patients with acute sarcoidosis (70 patients) were considered in NPL analysis of the *TLR4* locus, no significant results were gained, with *P*-values ranging from 0·17 to 0·39 for the four flanking microsatellite markers. However, with 83 chronic patients, *P*-values remained low (ranging from 0·02 to 0·05). When only patients with acute sarcoidosis were included in the analysis of the other TLR loci, the *TLR7/TLR8* locus on the X chromosome showed excess marker allele sharing (NPL score 1·3; $P = 0·01$) in affected siblings. NPL analysis with the subset of chronic patients showed no significant results for any other TLR locus.

Microsatellite TDTs

A total of 248 alleles of 28 microsatellite markers were used in the TDT. In the entire sib-pair family sample it revealed significant transmission distortion only for D3S3036 and D9S1776, besides several results with *P*-values smaller than 0·05 for single unusual alleles of other microsatellites. Marker D3S3026 resides on chromosome 3p, approximately 0·7 Mb

proximal of *TLR9*. It showed an increased transmission rate for one of seven alleles (transmitted/untransmitted = 20/10; $P = 0{\cdot}048$) and a decreased transmission rate for another allele (transmitted/untransmitted = 13/29; $P = 0{\cdot}014$). The D9S1776 locus is approximately 2·5 Mb centomeric to *TLR4* on chromosome 9q, and one of 10 alleles of this marker was less likely to be transmitted from heterozygous parents to affected offspring (transmitted/untransmitted = 17/37; $P = 0{\cdot}0064$). However, D9S1802 between *TLR4* and D9S1776 showed no transmission disequilibrium. Therefore the D9S1776 TDT result has possibly no meaning for the transmission of *TLR4* in the sib-pair families.

Single nucleotide polymorphism analyses in *TLR4*

Single nucleotide polymorphism analyses were subsequently performed at the only locus with significant linkage results, *TLR4*. The entire collection of patients, relatives and healthy control individuals were genotyped for four *TLR4* markers: c.-4036 A > G, c.94-837G > A, Asp299Gly and Thr399Ile. Extended samples containing unrelated sarcoidosis patients with Löfgren's syndrome, acute and chronic clinical forms of sarcoidosis were extracted from the genotype data pool and analysed separately. Genotype distributions in cases and controls corresponded to the expectations of Hardy–Weinberg equilibrium.

The size of the samples was sufficient to detect with a power of 0·8 an allelic disease association (carriership of the more rare SNP allele, significance level $\alpha = 0{\cdot}05$) with an odds ratio (OR) of 2·49, 2·00, 1·83 and 1·78 in the groups of unrelated patients with Löfgren's syndrome, acute sarcoidosis (including Löfgren's syndrome) chronic sarcoidosis and either acute or chronic sarcoidosis, respectively. With the exception of the Löfgren's syndrome sample, the size of the study populations was sufficient to detect an OR of 2·5 with a power of 0·95 or more. The observed allelic and genotype distributions of single markers in case–control comparison are outlined in Table 3. The allele and genotype frequency comparisons between Löfgren's syndrome, acute, chronic, both clinical phenotypes and controls for the analysed *TLR4* gene polymorphisms c.-4036A > G, c.94–837G > A, Asp299Gly and Thr399Ile did not reveal any significant associations. The G allele frequencies at the marker c.-4036A > G were almost identical in all study samples and ranged between 28% and 29%. The A allele at the downstream marker c.94–837G > A demonstrated similar results and ranged between 24% and 27%, not resulting in a significant difference between any of the patients' samples and controls. All study samples had a frequency of 6% of the allele G at the *TLR4* coding polymorphism Asp299Gly. The frequency of the allele T at the other coding SNP Thr399Ile was 6% in controls, 7% in acute, chronic or both phenotypes together and 8% in the group of patients with Löfgren's syndrome.

The results of the TDT are summarized in Table 4. The observed allele transmission rates of alleles at single markers c.-4036A > G, c.94-837G > A, Asp299Gly and Thr399Ile were not significant. There was no clear preference for the transmission of either allele at the c.-4036A > G polymorphism in Löfgren's syndrome, acute, chronic or both clinical phenotypes of sarcoidosis. Preferential transmission of the allele A at the marker c.94-837G > A, G at Asp299Gly and T at Thr399Ile was observed in all phenotypes analysed. In the four-marker haplotype analysis, five different haplotypes were transmitted from the parents to their affected children. The haplotype AGGT tagged by the allele G at the marker Asp299Gly was over-transmitted in all studied phenotypes, and reached a *P*-value of 0·07 (transmission rate 42/27) when acute and chronic families were analysed together. All other haplotypes were not significant in any of the studied clinical groups, and the transmission rates resulted in *P*-values greater than 0·1. As an additional finding we confirmed close linkage disequilibrium between the polymorphisms Asp299Gly and Thr399Ile [19,20]. The rare alleles of these two polymorphisms, G and T respectively, were transmitted together in 69 of 72 informative instances and resided on chromosomes further labelled by the alleles A and G of the upstream polymorphisms c.-4036A > G and c.94-837G > A.

Discussion

Toll-like receptors constitute a family of evolutionarily conserved transmembrane proteins that function as front-line pathogen recognition receptors of the innate immune defence system. They act as homodimers or heterodimers in the recognition of specific molecular determinants that represent characteristic components of infectious pathogens. These ligands include bacterial cell-surface LPS and lipoproteins, bacterial flagellin and fungal hyphae, double-stranded RNA of viruses and the unmethylated CpG islands of bacterial and viral DNA [8]. Some of these agents have been considered to be possibly involved in the aetiology of sarcoidosis, and a wide variety of other agents have also been accused [3–7]. Because of the wide spectrum of ligands of the different TLRs we decided to check whether any member of the TLR gene family is linked to the risk of sarcoidosis. Such a finding could help to focus on a distinct class of pathogens in the search for the cause of sarcoidosis.

So far, *TLR4* and more recently *TLR2* gene variations have been studied in sarcoidosis. An association between two intragenic non-synonymous *TLR4* point mutations, Asp299Gly and Thr399Ile and the risk to develop chronic sarcoidosis has been found in a study of 141 German patients and 141 controls [11]. However, this finding could not be replicated in two case–control association studies of 156 Dutch and 100 Greek patients [12,13]. It has been discussed that an accidentally low frequency of the presumed risk allele in the German control group contributed to the conflicting results [12]. A SNP at position -16934 of the promoter region of *TLR2* showed significant association with chronic

Table 3. Allele (a) and genotype (b) frequencies of the *TLR4* gene variants in patients with Löfgren's syndrome, acute and chronic clinical forms of sarcoidosis. One patient selected randomly from families with two or more patients of the same phenotype was chosen for the case–control comparisons. Sixty-five sib-pair families included patients with acute and with chronic sarcoidosis and contributed to both subgroups; *n*, number of analysed individuals.

TLR-4 polymorphism	Allele	Löfgren's syndrome $n = 152$	Acute (incl. Löfgren's syndrome) $n = 323$	Chronic sarcoidosis $n = 680$	Acute and chronic sarcoidosis $n = 938$	Control subjects $n = 537$
(a)						
c.-4036A > G	0	0	10	6	14	14
	A	220 (0·72)	453 (0·71)	981 (0·72)	1343 (0·72)	756 (0·71)
	G	84 (0·28)	183 (0·29)	373 (0·28)	519 (0·28)	304 (0·29)
c.94-837G > A	0	4	12	30	40	12
	A	78 (0·26)	150 (0·24)	346 (0·26)	464 (0·25)	282 (0·27)
	G	222 (0·74)	484 (0·76)	984 (0·74)	1372 (0·75)	780 (0·73)
Asp299Gly	0	2	6	8	14	
	A	283 (0·94)	600 (0·94)	1267 (0·94)	1741 (0·94)	1013 (0·94)
	G	19 (0·06)	40 (0·06)	85 (0·06)	121 (0·06)	61 (0·06)
Thr399Ile	0	2	12	12	26	20
	T	23 (0·08)	43 (0·07)	91 (0·07)	128 (0·07)	62 (0·06)
	C	279 (0·92)	591 (0·93)	1257 (0·93)	1722 (0·93)	992 (0·94)
(b)						
c.-4036 A > G	00	0	5	3	7	7
	AA	78 (0·51)	155 (0·49)	360 (0·53)	485 (0·52)	273 (0·51)
	AG	64 (0·42)	143 (0·45)	261 (0·39)	373 (0·40)	210 (0·40)
	GG	10 (0·07)	20 (0·06)	56 (0·08)	73 (0·08)	47 (0·09)
c.94–837G > A	00	2	6	15	20	6
	AA	8 (0·05)	14 (0·04)	47 (0·07)	56 (0·06)	42 (0·08)
	AG	62 (0·41)	122 (0·38)	252 (0·38)	352 (0·38)	198 (0·37)
	GG	80 (0·53)	181 (0·57)	366 (0·55)	510 (0·56)	291 (0·55)
Asp299Gly	00	1	3	4	7	0
	AA	132 (0·87)	282 (0·88)	592 (0·88)	813 (0·87)	479 (0·89)
	AG	19 (0·13)	36 (0·11)	83 (0·12)	115 (0·12)	55 (0·10)
	GG	0 (0·00)	2 (0·01)	1 (0·001)	3 (0·003)	3 (0·01)
Thr399Ile	00	1	6	6	13	10
	TT	1 (0·01)	3 (0·01)	1 (0·001)	5 (0·01)	3 (0·01)
	CT	21 (0·14)	37 (0·12)	89 (0·13)	118 (0·13)	56 (0·11)
	CC	129 (0·85)	277 (0·87)	584 (0·87)	802 (0·87)	468 (0·89)

sarcoidosis in patients from the Netherlands. However, this result could not be confirmed in a validation cohort of the same study [14].

We have applied a two-stage strategy including three types of raw data interpretation – NPL analysis, TDT and case–control association study – to test whether the TLR genes contribute to the increased inherited risk of sarcoidosis. NPL analysis and TDT do not require an independent matched control group, and do therefore not raise the issue of population stratification. Genetic linkage analysis of affected relatives is a most straightforward procedure to check extended segments of chromosomes for susceptibility gene candidates. TDT can be applied on the same genotype data set to test if a specific allele or haplotype within the shared chromosomal region is transmitted preferentially from parents to affected offspring, thus proving an association between the genetic marker and the risk of disease without the need of a control group.

The risk of sarcoidosis is increased in first-degree relatives of patients. According to different reports from European populations the sibling recurrence risk is approximately one in 100, or increased 20-fold compared with the prevalence in the general population [7]. Pedigrees with more than two affected members, as often seen in monogenic diseases with Mendelian inheritance, are rare in sarcoidosis, and complex multi-case families are unique exceptions [22]. These observations indicate that the effect of any single sarcoidosis susceptibility gene is presumably small, and that extended study groups are needed to demonstrate such genes, especially if the risk allele frequency is low as in the case of *TLR4* Asp299Gly. We have analysed extended groups of cases and controls for four *TLR4* polymorphisms – including two more common polymorphisms that ensured better coverage of the *TLR4* gene region and had a minor allele frequency of 0·25 or greater – to overcome the problem of insufficient power to

Table 4. Results of transmission disequilibrium tests (performed with GENEHUNTER version 2·1 [15]). Only one allele is listed for the four biallelic polymorphisms. Transmission rates are inverse and *P*-values identical for the second alleles. *P*-values of deviations from the expected ratio of trans : untrans are shown; trans: number of observed instances of allele or haplotype transmission from heterozygous parent to offspring with sarcoidosis; untrans: number of observed instances of allele or haplotype retention from heterozygous parent; single nucleotide polymorphisms (SNPs) in haplotypes are in the same order as listed above.

	Löfgren's syndrome		Acute sarcoidosis		Chronic sarcoidosis		Acute and chronic sarcoidosis	
TLR4 polymorphisms	Trans	Untrans	Trans	Untrans	Trans	Untrans	Trans	Untrans
Marker c.-4036A > G								
Allele G	26	36	61	58	113	127	174	185
	$P = 0{\cdot}20$		$P = 0{\cdot}78$		$P = 0{\cdot}37$		$P = 0{\cdot}56$	
Marker c.94–837G > A								
Allele A	40	31	67	62	103	91	170	153
	$P = 0{\cdot}29$		$P = 0{\cdot}66$		$P = 0{\cdot}39$		$P = 0{\cdot}34$	
Marker Asp299Gly								
Allele G	9	5	16	11	35	24	51	35
	$P = 0{\cdot}29$		$P = 0{\cdot}34$		$P = 0{\cdot}15$		$P = 0{\cdot}084$	
Marker Thr399Ile								
Allele T	10	5	17	11	34	26	51	37
	$P = 0{\cdot}20$		$P = 0{\cdot}26$		$P = 0{\cdot}30$		$P = 0{\cdot}14$	
Four marker haplotype								
Haplotype A A A T	1	0	1	0	1	1	2	1
	$P = 0{\cdot}32$		$P = 0{\cdot}32$		$P = 1$		$P = 0{\cdot}56$	
Haplotype A A A C	30	21	52	44	76	66	128	110
	$P = 0{\cdot}21$		$P = 0{\cdot}41$		$P = 0{\cdot}40$		$P = 0{\cdot}24$	
Haplotype A G A C	29	32	46	61	100	108	146	169
	$P = 0{\cdot}70$		$P = 0{\cdot}18$		$P = 0{\cdot}58$		$P = 0{\cdot}20$	
Haplotype A G G T	8	4	13	9	29	18	42	27
	$P = 0{\cdot}23$		$P = 0{\cdot}39$		$P = 0{\cdot}11$		$P = 0{\cdot}071$	
Haplotype G G A C	17	28	46	44	74	87	120	131
	$P = 0{\cdot}10$		$P = 0{\cdot}83$		$P = 0{\cdot}31$		$P = 0{\cdot}49$	

detect association. Haplotypes were used only if they could be derived from family genotype information.

Microsatellite analysis showed significant results only at the *TLR4* gene locus both in NPL analysis and TDT. The inclusion of microsatellite marker D9S934 from the whole genome marker set [10] into the present study made comparison possible of both linkage analyses. While the NPL score at D9S934 in the previous study of 63 families was 1·82 ($P = 0{\cdot}034$), it increased to 2·41 ($P = 0{\cdot}008$) with the inclusion of 20 additional sib-pair families. This increase of significance with additional families underlines the relevance of this locus in the search for sarcoidosis susceptibility genes. In the only other genome-wide linkage scan in sarcoidosis, based on African American families, a peak on the long arm of chromosome 9 was one of the two coinciding peaks of both whole genome studies [23]. This peak was labelled in the African American data set mainly by the microsatellite D9S1825. However, as this marker is located approximately 7·4 Mb telomeric to *TLR4* and D9S1864, no further conclusions can be drawn without closer and overlapping linkage information from the African American families sample.

The *TLR4* gene was chosen for subsequent SNP analysis in a high throughput genotyping setting, but revealed no significant result in the following case–control comparisons. This outcome supported the assumption derived from Dutch and Greek studies that no association exists between *TLR4* Asp299Gly and the risk to develop sarcoidosis or to proceed to chronic sarcoidosis. The minor allele frequency of *TLR4* Asp299Gly in our German control group (0·056) is close to the frequency in the control group from the Netherlands (0·063) [12]. It differs grossly from the frequency found in the German control group communicated by Pabst *et al.* (0·028) [11]. This appears to support the assumption that an accidentally low allele frequency in the control group of Pabst *et al.* contributed to significant differences between the chronic sarcoidosis group and controls in their study. Recently, a study about the worldwide distribution of *TLR4* Asp299Gly and Thr399Ile has been published that included 632 and 209 healthy individuals from Germany and the Netherlands, respectively [24]. The minor Asp299Gly allele frequency was 0·057 in the German group and 0·072 in the Dutch group. One of the largest study groups to analyse the Asp299Gly polymorphism has been recruited at five different places in western Germany [25]. It included 3000 individuals and has been designed as a prospective community-based study that represents the local German population. In

this large sample was the minor Asp299Gly allele frequency 0·04 and thus closer to the frequency in the control group of Pabst *et al*.

In summary, we are left with conflicting results: linkage studies point to the TLR-4 locus on chromosome 9, but case–control association studies largely exclude the functional polymorphism Asp299Gly and three others from having a meaning in sarcoidosis. However, our TDT results of *TLR4* SNP haplotypes showed that the only haplotype with the presumed risk allele of Asp299Gly was transferred more often than expected by chance from heterozygous parents to offspring who developed sarcoidosis. As a consequence of the low frequency of the *TLR4* Asp299Gly polymorphism, only 69 parents of the study population were heterozygotes and contributed information to the test. On 42 of 69 occasions the presumed risk allele was transferred, on 27 occasions the other allele was transferred ($P = 0{\cdot}07$). In the absence of evidence that *TLR4* Asp299Gly is associated directly with the risk of sarcoidosis, the distorted transmission could be explained by an extended haplotype that carries the real risk allele and is labelled by *TLR4* Gly299. This hypothetical susceptibility allele could be located outside the coding sequence of *TLR4*, possibly affecting regulatory elements of the gene. Interestingly, a comparable finding was reported by Oostenbrug *et al*. [26], who searched for association between *TLR4* polymorphisms and inflammatory bowel disease (IBD) after conflicting reports on association between Asp299Gly and the risk of IBD have been published. IBD and sarcoidosis have in common a complex multi-organ inflammation and the formation of granulomas. Oostenbrug *et al*. genotyped 781 IBD cases and 315 controls from the Netherlands for Asp299Gly and Thr399Ile and for four flanking microsatellite markers, including D9S1864. No association was found between both SNPs and the risk of IBD, but significant microsatellite haplotype sharing was observed in the patients' group. This effect was due mainly to the contribution of D9S1864 to the risk haplotypes, which otherwise carried the common alleles of Asp299Gly and Thr399Ile. The authors proposed that IBD susceptibility is associated with other variants of *TLR4.* Given the similarity of our results, this proposed susceptibility could be linked to a common inflammatory feature of IBD and sarcoidosis.

Alternatively, another gene in the vicinity of *TLR4* could be responsible for the NPL peak and positive TDT result of our study. Genes from this chromosomal region on the long arm of chromosome 9 have been studied for association with sarcoidosis before. In fact, one of the first genetic studies in sarcoidosis focused on the ABO blood groups and found increased risk of sarcoidosis associated with blood group A in 518 sarcoidosis patients [27]. However, the ABO gene locus resides approximately 15 Mb towards the telomere of chromosome 9q and is therefore most probably too far away to account for the linkage peak at the *TLR4* locus (all positions and distances according to NCBI Map Viewer, build 36·2). By contrast, the *ORM1* gene that codes for the acute phase protein, orosomucoid, is located closer to the linkage peak region of this study, at a distance of approximately 3·4 Mb. Association of the allele ORM*1 with sarcoidosis has been communicated from a study of Swedish patients [28]. At least two other reasonable functional candidate genes are located close to peak at the *TLR4* gene locus, *TNFSF15* (3·5 Mb from *TLR4*) and tumour necrosis factor receptor-associated factor 1 (*TRAF1*) (3·2 Mb from *TLR4*). *TNFSF15* is a member of the tumour necrosis factor super-family. Haplotypes of this gene have shown a highly significant association with Crohn's disease in Japanese and European study populations [29]. *TRAF1* is involved in the regulation of NF-kappa B activity, which plays a crucial role in sarcoidosis [9,30].

From the study of all seven TLR gene loci presented here, we conclude that the *TLR4* gene and its chromosomal vicinity appear to be a promising target region for further efforts in the search for sarcoidosis susceptibility genes.

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