

Study of C-C Chemokine Receptor 2 Alleles in Sarcoidosis, with Emphasis on Family-based Analysis

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Prevailing models of sarcoidosis pathogenesis involve the activation of alveolar macrophages, aggregation of CD4⁺ T lymphocytes, and their accumulation in epithelioid cell granulomas. Increasing evidence suggests that each of these steps is modified by the host genetic constitution. Consequently, candidate susceptibility genes have been selected based on their potential function under this model. The C-C chemokine receptor 2 (CCR2) is involved in Th1 immune activity by recruiting competent cells and possibly by balancing response. CCR2 gene variants have been shown to be associated with sarcoidosis or, more specifically, with Löfgren's syndrome, a distinct form of acute sarcoidosis. We have studied three CCR2 gene polymorphisms (c.190G>A, c.840C>T, and c.4385A>T) in an extended sample of 1,203 patients with sarcoidosis and their relatives. Case-control comparisons and family-based genetic analyses did not support previous findings of an association between CCR2 gene variability and the risk of sarcoidosis. However, they confirmed linkage disequilibrium and showed positive linkage results ($p = 0.034$) and therefore suggest a susceptibility gene in the surrounding chromosomal region.

Keywords: genetic predisposition to disease; genotype; Löfgren's syndrome; transmission disequilibrium test

Sarcoidosis is a multiorgan inflammatory disease of unknown cause that primarily affects the lung and the lymphatic systems, but can afflict almost every other organ of the body. The disorder is characterized by an exaggerated cellular immune reaction that is believed to be triggered by so far unidentified, but presumably inhaled, exogenous agents (1, 2). The prevailing model of sarcoidosis pathogenesis involves activation of alveolar macrophages, aggregation of CD4⁺ T lymphocytes, and the accumulation of these cells in epithelioid cell granulomas, which are typical at the sites of inflammation (3, 4).

Clinical presentation of the disorder varies widely, with two main phenotypes: acute and chronic sarcoidosis. Acute sarcoidosis is predominantly associated with a self-limited course of disease and a favorable prognosis. A subset of patients with acute disease present with symptoms of Löfgren's syndrome, which are typically a combination of fever, bilateral hilar lymphadenopathy, erythema nodosum, and arthritis, often of the ankle joints. In contrast, chronic sarcoidosis tends to start with subtle, creeping symptoms and has a considerable risk of proceeding to enduring inflammation and subsequent organ damage (1, 2, 4).

Inherited susceptibility to sarcoidosis is well documented through differing prevalence rates in different populations and through an increased risk of occurrence in close relatives of patients (5–7). The spectrum of clinical presentation and pattern of organ involvement differs between ethnic groups (8, 9). A complex blend of predisposing and protective gene variants is believed to contribute to an individual's susceptibility to sarcoidosis, as well as to the various presentations and prognoses of the disease. Major candidate gene variants conferring susceptibility or resistance to sarcoidosis have been identified in the HLA gene cluster of chromosome 6 using case-control study designs and genetic linkage analyses (10–17). However, these gene effects are not sufficient to explain the approximately 20-fold increased manifestation risk in close relatives of patients with sarcoidosis, as observed in European populations (18, 19).

In the search for additional predisposing genes, candidate genes have been selected for analysis because of their potential function in the pathogenesis of sarcoidosis, with a focus on antigen recognition, processing, and elimination. Among these functional candidate genes, the C-C chemokine receptor 2 (CCR2) has been studied in different settings with remarkable results. The CCR2 gene is located on the short arm of chromosome 3 (cytogenetic band 3p21). A nonsynonymous polymorphism in exon 1 (c.190G>A) leads to the substitution of valine by isoleucine (p.V64I) in the transmembrane region of the protein. A decreased risk of sarcoidosis in carriers of the rarer allele, c.190A (p.64I), has been described in Japanese patients (20). This was not replicated in a sample of Czech patients, even though there was a nominal, but not significant, excess of c.190A in control subjects (21). Recently, a set of eight single nucleotide polymorphisms of the CCR2 gene, including c190G>A, has been analyzed in a Dutch sarcoidosis case-control study (22). The superior resolution of the haplotype analysis enabled the demonstration of an association between one specific CCR2 haplotype and an increased risk of Löfgren's syndrome. The fact that the CCR2 gene locus on the short arm of chromosome 3 (3p21) coincides with a linkage peak in a genomewide search for predisposing loci (23) further contributes to the candidate status of CCR2 gene variants in the etiology of sarcoidosis. Here, we contribute novel association and genetic linkage data for CCR2 alleles from an extended sample of patients with sarcoidosis and their families.

METHODS

Patients, Families, and Control Subjects

Patients were actively contacted through the German Sarcoidosis Patients' Organization (Deutsche Sarkoidose-Vereinigung e.V.; www.sarkoidose.de), specialized hospitals, and practitioners, and by calls for participation in the study that were published via health insurance institutions. Parents were included when possible. Patients from families with two or more affected members were interviewed by telephone about their sarcoidosis history and family structure.

All patients completed a questionnaire on the course of disease. Patients' physicians were contacted to confirm the diagnosis and to

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provide records. The average age at diagnosis was 36.4 years (SD, 10.9 years) and ranged from 12 to 80 years. Sarcoidosis was confirmed by biopsy in 1,001 of 1,203 (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. The control group comprised 545 healthy blood donors recruited through the Department of Transfusion Medicine at Kiel University Hospital, Kiel, Germany. The control subjects consisted of 268 (49.1%) males and 277 (50.9%) females with an average age at inclusion of 43 years (SD, 12.7 years). All families with two or more patients were of German descent. All other patients and relatives were German residents. All participants gave written, informed consent before inclusion in the study. Study protocols were approved in writing by the institutional ethics and data protection authorities.

Patients were classified according to disease presentation on the basis of all available information (questionnaires completed by patients and physicians, hospital records, and interview information for familial cases). The entire sarcoidosis sample consisted of 1,203 patients and 1,084 close relatives (mainly parents, without sarcoidosis). Three hundred and seventy patients had acute sarcoidosis, with sudden complaints and recovery within 2 years. One hundred and sixty-five of these patients with acute sarcoidosis exhibited the characteristic combination of symptoms of Löfgren's syndrome (bilateral hilar lymphadenopathy, erythema nodosum, and arthropathy). Seven hundred and thirty-nine patients suffered from chronic sarcoidosis. The majority of these exhibited subtly intensifying early symptoms followed by enduring disease activity for 2 years or longer. The remaining 94 patients showed other phenotypes (e.g., solely cutaneous sarcoidosis), or were detected serendipitously by radiography for other reasons and had no specific complaints. These individuals and their relatives were genotyped but excluded from further data analysis with the exception of the linkage disequilibrium analysis. An overview of the study population of patients with sarcoidosis is given in Table 1.

CCR2 Genotyping

Three polymorphic sites of the CCR2 gene—c.190G>A (p.V64I, rs1799864), c.840C>T (p.N260N, rs1799865), and c.4385 A>T (rs1034382, in the downstream intergenic sequence)—were genotyped using Taqman (24) and TaqMan-MGB (25) assays. Ready-to-use Assay-on-Demand and Assay-by-Design assays were used for typing c.840C>T and c.4385T>A, respectively (all assays were from Applied Biosystems, Foster City, CA; www.store.appliedbiosystems.com). For the c.190G>A variant, a TaqMan assay was designed manually using the Primer Express 2.0 software (Applied Biosystems). Assay details are given in Table 2.

Data Analysis

Marker genotypes were tested for Hardy-Weinberg equilibrium in the case and control study populations. Allele and genotype frequencies in cases and control subjects were compared using Pearson's χ^2 test of the SPSS statistical software package (SPSS, Inc., Chicago, IL). Markers in families and trios (single patients with parents) were evaluated for distorted transmission using the transmission disequilibrium test of the Genehunter 2.1 program (26). The same program was used for nonparametric linkage analysis and haplotype reconstruction in families. The

allelic correlation coefficient r^2 was computed as an index of the pairwise linkage disequilibrium between markers (27).

RESULTS

The entire collection of 2,832 samples (1,203 patients, 1,084 relatives, and 545 control individuals) was genotyped for three single nucleotide polymorphisms in a high-throughput setting. The overall genotype calling rate was 0.98. The observed allele and genotype frequencies of the three CCR2 polymorphisms in the sarcoidosis patient study population (323 patients with acute sarcoidosis and 680 patients with chronic sarcoidosis, after selecting a single patient at random from families with two or more patients of the same type) and control individuals are listed in Table 3. Genotype distributions in cases and control subjects corresponded to the expectations of Hardy-Weinberg equilibrium, with the exception of c.190G>A genotypes in cases ($p = 0.018$). This mainly resulted from a lack of AA homozygotes (observed 1, expected 6.5). The preferential drop-out of AA genotypes does not appear likely if drop-out genotypes are reconstructed from family data. The random exclusion of one AA homozygote from a family with two patients may have contributed to the low frequency of AA homozygotes but cannot fully explain it. There were no significant ($p < 0.1$) differences between any sample of patients (Löfgren's syndrome; acute sarcoidosis, including Löfgren's syndrome; chronic sarcoidosis; acute and chronic sarcoidosis) and the control group or between different samples of patients. There was a strong linkage disequilibrium between c.840C>T and c.4385A>T with an r^2 value of 0.72 in the control population. In the families, the alleles of the three markers cosegregated with no evident haplotype recombination based on 527 meioses. Three hundred and sixty-two unequivocal haplotype transmissions could be derived from the pedigree and genotype data. Only four of eight possible haplotypes occurred with notable frequencies, and one rare haplotype segregated in one family, but was not transmitted to the patient, who had Löfgren's syndrome. The observed transmission rates of alleles and haplotypes are given in Table 4. There was no significant ($p < 0.05$) transmission disequilibrium in the study population or in any of the subsets. A suggestive transmission disequilibrium ($0.05 < p < 0.1$) for two haplotypes was observed in the patients with acute sarcoidosis. One of these distortions was also evident in the subset of patients with Löfgren's syndrome. These haplotypes were divergent with respect to the c.190G>A polymorphism: the c.190A allele (corresponding to p.64I) was overtransmitted and the c.190G allele (corresponding to p.64V) was undertransmitted to patients with sarcoidosis.

Ninety-three families with two or more affected siblings were available for genetic linkage analysis. These showed significant

TABLE 1. DETAILS OF THE STUDY POPULATION OF PATIENTS WITH SARCOIDOSIS

Family Structure	No. Families	No. Patients	Male Patients	Female Patients	Löfgren's Syndrome	Acute Sarcoidosis*	Chronic Sarcoidosis	Other Forms 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, 2° or 3° relatives	24	48	28	20	6	17	25	6
More than two patients	23	73	38	35	12	30	39	4
Total	997	1,203	503	700	165	370	739	94

Patients include those with acute sarcoidosis (sudden complaints and recovery within 2 years), Löfgren's syndrome (acute sarcoidosis with bilateral hilar lymphadenopathy, erythema nodosum, and arthropathy), or chronic sarcoidosis (stealthy beginning, enduring disease activity for 2 years or longer).

* Including Löfgren's syndrome.

TABLE 2. PRIMER AND PROBE SEQUENCES OF CCR2 TYPING ASSAYS, USING THE TAQMAN TECHNIQUE

Marker	Primers	Probes
c.190G>A	5'- CCGCTCTACTCGCTGGTGT 5'-GTTGAGCAGGTAATGTCAGTCAA	TET-CAACATGCTGGTCATCCTCATCTTAATAAACT FAM-CAACATGCTGGTCGTCCTCATCTTAATAAACT
c.4385A>T	5'- ACATTTGAGTAGGCTGTTCCAGT 5'- CTTCAGACTCATCCCTCAGAATG	VIC- CAGGTGGAAGATGATGA FAM- CAGGTGGAAGTTGATGA

For c.840C>T, the assay was obtained as Assay-on-Demand from Applied Biosystems (www.appliedbiosystems.com); no primer and probe information is available. Flanking sequences are provided at http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=1799865.

($p = 0.034$) concordance of affected sib pairs for at least one parental CCR2 haplotype, as revealed by nonparametric multipoint linkage (NPL) analysis (NPL score, 1.78). There were no significant results when only chronic patients (NPL score, 0.59; $p = 0.12$) or only acute patients (NPL score, 0.08; $p = 0.43$) were considered. We could extract 75 independent informative pairs of affected siblings from the set of families (one pair randomly chosen from families with more than two affected siblings). Seventy-three of these 150 patients showed acute sarcoidosis and 77 suffered from chronic sarcoidosis. Twenty-four sib pairs were concordant for the acute sarcoidosis phenotype and 26 sib pairs for chronic sarcoidosis, whereas the remaining 25 pairs of affected siblings exhibited discordant sarcoidosis phenotypes. In 9 of 24 sib pairs concordant for acute sarcoidosis, both patients had Löfgren's syndrome. In three of these nine families, both patients had at least one copy of the c.4385T allele, and in five families, both patients were negative. In two families with Löfgren's syndrome present in one of two patients, the patients were discordant for the c.4385T allele carrier status. In

both families, the patients with Löfgren's syndrome were negative for the putative Löfgren's syndrome risk allele and the patient without Löfgren's syndrome was positive.

DISCUSSION

Sarcoidosis is an inflammatory disease with a diverse clinical presentation. The disorder appears to develop gradually, through the interaction of some exogenous trigger and the immune system of a susceptible host. In fact, the presumed initial stage of the process, bilateral hilar lymphadenopathy, appears to be relatively common, with an adult lifetime risk of 1 in 85 for women and 1 in 105 for men, as derived from a 15-year longitudinal study in Sweden (28). It is not clear which attributes of the patients influence the progression of the disease from the inflammatory reaction to the persistence of specific granulomas in a minority of affected people. However, it has been suggested by previous publications that variants in several genes, especially in the major histocompatibility gene complex, are associated with

TABLE 3. ALLELE AND GENOTYPE FREQUENCIES OF THREE CCR2 GENE POLYMORPHISMS, c.190G>A (p.V64I, rs1799864), c.840C>T (p.N260N, rs1799865), AND c.4385A>T (rs1034382, IN THE DOWNSTREAM INTERGENIC SEQUENCE) IN PATIENTS WITH SARCOIDOSIS* AND CONTROL SUBJECTS

CCR2 Polymorphism	Allele (nucleotide)	Löfgren's Syndrome (n = 153)	Acute (incl. Löfgren's syndrome) (n = 323)	Chronic Sarcoidosis (n = 680)	Acute and Chronic Sarcoidosis (n = 938) [‡]	Control Subjects (n = 545)
Alleles						
c.190G>A	0 [†]	6	14	46	54	36
	c.190A	21 (0.07)	48 (0.08)	121 (0.09)	154 (0.08)	82 (0.08)
	c.190G	279 (0.93)	584 (0.92)	1193 (0.91)	1668 (0.92)	972 (0.92)
c.840C>T	0 [†]	4	12	6	12	58
	c.840C	103 (0.34)	213 (0.34)	427 (0.32)	603 (0.32)	333 (0.32)
	c.840T	199 (0.66)	421 (0.66)	927 (0.68)	1261 (0.68)	699 (0.68)
c.4385A>T	0 [†]	2	10	20	26	44
	c.4385A	223 (0.73)	473 (0.74)	1016 (0.76)	1397 (0.76)	804 (0.77)
	c.4385T	81 (0.27)	163 (0.26)	324 (0.24)	453 (0.24)	242 (0.23)
Genotypes						
c.190G>A	00 [†]	3	7	23	27	18
	AA	0	0	2 (0.003)	1 (0.001)	1 (0.002)
	AG	21 (0.14)	48 (0.15)	117 (0.18)	152 (0.17)	80 (0.15)
	CG	129 (0.86)	268 (0.85)	538 (0.82)	758 (0.83)	446 (0.85)
	GG					
c.840C>T	00 [†]	2	6	3	6	29
	CC	19 (0.12)	37 (0.12)	73 (0.11)	106 (0.12)	56 (0.11)
	CT	65 (0.43)	139 (0.44)	281 (0.41)	391 (0.42)	221 (0.42)
	TT	67 (0.44)	141 (0.44)	323 (0.48)	435 (0.47)	239 (0.46)
	TT					
c.4385A>T	00 [†]	1	5	10	13	22
	AA	81 (0.53)	176 (0.55)	386 (0.58)	529 (0.57)	309 (0.59)
	AT	61 (0.40)	121 (0.38)	244 (0.36)	339 (0.37)	186 (0.36)
	TT	10 (0.07)	21 (0.07)	40 (0.06)	57 (0.06)	28 (0.05)
	TT					

There were no significant ($p < 0.1$) differences between any sample of patients (Löfgren's syndrome, acute sarcoidosis [including Löfgren's syndrome], chronic sarcoidosis, acute and chronic sarcoidosis) and the control group or between different samples of patients.

* One patient selected randomly from families with two or more patients of the same type.

[†] 0 and 00: due to genotype drop out, not considered in frequencies.

[‡] As a consequence of random selection of only one patient per family, the number of patients in this sample is smaller than the sum of patients in the samples of acute and chronic sarcoidosis.

TABLE 4. RESULTS OF THE TRANSMISSION DISEQUILIBRIUM TEST

CCR2 Polymorphism Alleles/Haplotypes	Löfgren's Syndrome		Acute Sarcoidosis		Chronic Sarcoidosis		Acute and Chronic Sarcoidosis	
	trans*	untrans†	trans	untrans	trans	untrans	trans	untrans
Marker 1: c.190G>A Allele c.190A	9	8	19	12	42	54	61	66
	p = 0.81		p = 0.21		p = 0.22		p = 0.66	
Marker 2: c.840C>T Allele c.840C	35	27	59	52	119	126	178	178
	p = 0.31		p = 0.51		p = 0.65		p = 1.00	
Marker 3: c.4385A>T Allele c.4385A	22	28	43	48	103	94	146	142
	p = 0.40		p = 0.60		p = 0.52		p = 0.81	
Markers 1 and 2 and 3								
Haplotype: A C A	0	1	0	1			0	1
Haplotype: A T A	9	5	17	8 [§]	36	44	53	52
Haplotype: G C A	12	7	21	15	26	28	47	43
Haplotype: G C T	23	19	37	34	74	76	111	110
Haplotype: G T A	20	32 [‡]	39	56	112	100	151	156

Only one allele is listed for the three biallelic polymorphisms. Transmission rates are inverse and p values identical for the second alleles. The p values of deviations from the expected ratio of trans to untrans are shown for the three polymorphisms. The p values for haplotype transmissions were $p > 0.1$ unless otherwise indicated.

* 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.

‡ p = 0.09.

§ p = 0.07.

|| p = 0.08.

the development of either acute or chronic sarcoidosis (10–12, 15, 29–31).

CCR2 could be another effective gene in fine-tuning the granulomatous immune reaction. The gene product is expressed on the surface of macrophages, monocytes, dendritic cells, and T lymphocytes, and is a receptor of monocyte chemotactic protein 1 and related chemokines. It is involved in inflammatory cell recruitment, and CCR2 knockout mice are unable to initiate immune control of *Mycobacterium tuberculosis* infection (32). Recently, a dual role of CCR2, influencing subsequent downregulation of the inflammatory reaction, has been discussed (33).

Three previous reports from Japanese (20), Czech (21), and Dutch (22) study populations have communicated the results of CCR2 c.190G>A genotyping in the search for association with sarcoidosis. A significantly reduced allele frequency of 12.5% for the rarer allele (c.190A) was found in 100 patients from Japan as compared with a frequency of 26.2% in 122 healthy control subjects ($p = 0.00072$; odds ratio [OR], 0.372; 95% confidence interval [CI], 0.209–0.666) (20). Underrepresentation of the same variant, c.190A, was observed in a study of 65 Czech patients and 80 control subjects, but this did not achieve significance (c.190A frequencies: 6.9% in cases and 11.9% in control subjects, $p = 0.17$) (21). In the third, more recent, publication, c.190A allele frequencies were similar (~ 6%) in 137 Dutch cases (47 patients with Löfgren's syndrome and 90 patients with non-Löfgren's sarcoidosis) and 167 control subjects. When only patients with Löfgren's syndrome were considered, the c.190A allele frequency was only marginally reduced to 5% (22). The observed c.190A allele frequencies of 8% in the 938 German patients with sarcoidosis and 545 control subjects in our study ($p = 0.53$; OR, 1.09; CI, 0.83–1.45) do not support the association between this allele and a reduced risk of sarcoidosis, as has been reported for Japanese and Czech patients (20, 21). Rather, our results confirm the findings from the Dutch study populations (22), although the frequency of the rarer allele is slightly higher in our cases and control subjects (8%) when compared with the Dutch samples (5–6%).

However, Spagnolo and colleagues (22) could demonstrate, by the simultaneous analysis of seven additional polymorphisms

across the CCR2 gene, a significantly increased carrier frequency of one haplotype, not bearing the c.190A allele, in Dutch patients with Löfgren's syndrome (74%) when compared with patients without Löfgren's syndrome (carrier frequency, 38%; $p < 0.0001$; OR, 4.80; CI, 2.2–10.5) or when compared with control subjects (carrier frequency, 38%; $p < 0.0001$; OR, 4.81; CI, 2.3–10.0). Furthermore, this major Löfgren's syndrome risk haplotype was clearly distinct from the eight other haplotypes found in cases and control subjects, bearing the alternate alleles at four of the eight polymorphic sites. Our results confirm the strong linkage disequilibrium among the CCR2 polymorphisms and the single nucleotide polymorphism allele c.4385T is identical with one of the alleles indicative of the Löfgren's syndrome risk haplotype in Dutch patients. Therefore, our results can be compared—with limitations—to the haplotype data of Spagnolo and coworkers (22).

There was a significantly increased c.4385T allele frequency in Dutch patients with Löfgren's syndrome (44%) when compared with patients with non-Löfgren's sarcoidosis (23%; $p = 0.008$, corrected for multiple testing) and with control subjects (21%; $p = 0.0008$, corrected for multiple testing) (22). In contrast, we did not observe significantly ($p < 0.1$) different c.4385T allele frequencies in German control subjects (23%) and cases (24%), irrespective of stratification for chronic (24%) or acute (26%) sarcoidosis or Löfgren's syndrome (27%; $p = 0.21$; OR, 1.21; CI, 0.90–1.62 when compared with the healthy control sample). The same was true when genotype frequencies were considered instead of allele frequencies. Seventy-one patients of the Löfgren's syndrome sample (47%) and 214 individuals of the control group (41%) were heterozygote or homozygote carriers of c.4385T ($p = 0.20$; OR, 1.27; CI, 0.88–1.82). Consequently, regarding individual CCR2 gene polymorphisms, including c.4385A>T, which tags the Löfgren's syndrome risk haplotype reported by Spagnolo and coworkers (22), we are unable to confirm significant differences between control subjects and cases with sarcoidosis or sarcoidosis subtypes in our German study populations. The sample size in the present study greatly exceeds that in the previous three studies described previously and therefore our inability to detect significant association is unlikely to be caused by lack of power. Our sample has 100%

power to detect the allele OR of 2.87 reported by Spagnolo and colleagues (22) for the association between Löfgren's syndrome and c.4385A>T (at $\alpha = 0.05$ and a risk-factor frequency of 0.24 [present data]). Furthermore, our sample has 100% power to detect both the OR of 0.37 reported by Hizawa and coworkers (20) and the OR of 0.52 reported by Petrek and coworkers (21) for carriage of the c.190A allele in patients with sarcoidosis (acute and chronic; at $\alpha = 0.05$ and a risk-factor frequency of 0.17 [present data]).

One reason for conflicting results in the field of association studies could be undetected bias of the control sample. To overcome this problem, family-based methods, such as the transmission disequilibrium test, have been developed that work without a control sample (26). The transmission disequilibrium test counts the number of transmissions of parental gene variants to affected offspring. Deviation from random transmission argues for a predisposing effect of the more frequently transmitted allele. The transmission disequilibrium test can be applied to individual polymorphisms as well as to strings of adjacent polymorphisms, thus analyzing the transmission of haplotypes. In our families, the c.4385T allele segregated exclusively with c.190G (see Table 4), thus confirming strong linkage disequilibrium between both loci as found in the Dutch study populations. The c.4385T allele is included in, and tags, the Löfgren's syndrome risk haplotype communicated by Spagnolo and coworkers (22). In the informative families of our study population, the equivalent haplotype (c.190G – c.840C – c.4385T) is in almost perfect transmission equilibrium, being transmitted from heterozygous parents to patients on 111 of 221 occasions. The transmission of the putative risk haplotype to patients with acute sarcoidosis or Löfgren's syndrome was not significantly more frequent than expected, with transmission rates of 37 in 71 (expected 35.5, $p = 0.72$) and 23 in 42 (expected 21, $p = 0.53$), respectively.

In summary, we cannot confirm previous reports that suggest a significant impact of CCR2 alleles on the risk, or on the presentation, of sarcoidosis. The scope and the structure of our samples, with the majority of patients being suitable for family-based analyses, facilitated the use of robust statistics. However, an obvious disadvantage of a recruitment strategy that is based on calls for participation is the risk of ascertainment bias, whereby severe and unusual cases may be overrepresented. Furthermore, approximately two of three patients without a family history of sarcoidosis suffered from chronic sarcoidosis, even though this phenotype is clearly less common than this among patients with sarcoidosis in Germany. In the set of families with two or more patients, one of two patients exhibited chronic sarcoidosis. However, considering the markedly wide spectrum of sarcoidosis presentation, any hospital-based study using consecutive patients would be prone to underrepresentation of less severe cases.

In addition to ascertainment bias, stratification for sarcoidosis phenotypes could be hampered by inaccuracies. In our study, this classification is based on various sources, including reports from hospitals, doctors, and patients. Certainly there is a risk of incomplete and misleading information. On the other hand, it appears doubtful that Löfgren's syndrome represents a clear-cut segment of the sarcoidosis patient population. The course of disease and its outcome is specific to, and to some extent predictable in, the majority of patients with Löfgren's syndrome. However, a portion of these cases will develop chronic sarcoidosis, sometimes after an interval of many years (34). Twenty-five of 75 informative pairs of affected siblings in this study exhibited discordant acute or chronic sarcoidosis phenotypes. This argues against acute sarcoidosis being a distinct heritable sarcoidosis phenotype. The examination of nine families with siblings who suffered from Löfgren's syndrome did not support a role for the

c.4385T allele in the etiology of Löfgren's syndrome; however, this subset was too small for a meaningful statistical analysis.

The Dutch control subjects of Spagnolo and coworkers (22) and both the German patients and control subjects in the current study exhibit similar CCR2 allele and haplotype frequencies (with c.4385T representing the Löfgren's risk haplotype of Spagnolo and coworkers). The definition of the Löfgren's syndrome phenotype is congruent in both studies. It seems unlikely that the German Löfgren's sample includes a notable proportion of patients with non-Löfgren's sarcoidosis. We cannot exclude that we have missed some patients with Löfgren's syndrome in the acute sarcoidosis sample as a result of incomplete information, but this would not explain the lack of association between our Löfgren's syndrome sample and c.4385T. Perhaps Dutch patients with Löfgren's syndrome are different from German patients and Dutch and German control subjects because of regional differences in the composition of exogenous triggers. The Dutch patients (all from one hospital?) presumably originate from a more homogeneous environment than the German patients, who came from all over Germany. In addition, historical trigger variables may be important because some of the German patients suffered and recovered from Löfgren's syndrome more than 20 years ago.

Our results provide little support for the assertion that variants of the CCR2 gene itself contribute significantly to the etiology of sarcoidosis. However, the inclusion of 93 families in our study containing siblings suffering from sarcoidosis permits us to address the more general question of whether the chromosomal segment that carries the CCR2 gene sequence is linked to the risk of sarcoidosis. A positive NPL score of 1.7 ($p = 0.034$) indicated an excess of haplotype sharing in affected sib pairs, and was the most pronounced result from different types of analyses presented here. This positive linkage result is in concordance with data from a previous genomewide linkage analysis for sarcoidosis (23) that included 10 highly polymorphic microsatellite markers, evenly distributed over the short arm of chromosome 3. The scan was based on 63 of the 93 families of the current set and revealed a linkage peak (NPL score, 2.39; $p = 0.009$) at the microsatellite marker D3S1766 locus. This is located approximately 5 Mb closer to the centromere of chromosome 3 than CCR2. The lower NPL score of the present study despite a larger number of analyzed families could be explained by the lower information content of the biallelic single nucleotide polymorphisms when compared with multiallelic microsatellite markers. Genetic linkage results decrease with increasing distance between the analyzed marker and the disease gene. Therefore, our linkage result could also mean that the CCR2 polymorphisms under study are located close to, but not exactly at the site of, the real predisposing gene in this chromosomal region.

Conflicting results between CCR2 investigations in sarcoidosis could be explained by linkage disequilibrium between CCR2 gene variants and polymorphisms in a neighboring sarcoidosis susceptibility gene. Linkage disequilibrium within the CCR2 gene has been reported by Spagnolo and colleagues (22) and could be confirmed by our data. Linkage disequilibrium between polymorphic genes within this chromosomal segment has also been documented (35). Containing a number of other reasonable candidate genes, including a cluster of additional chemokine receptors, macrophage-stimulating protein, or MST1, and its receptor, and Toll-like receptor 9, the short arm of chromosome 3 remains a promising target in the search for sarcoidosis susceptibility genes.

Conflict of Interest Statement: R.V. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; P.J.P.C. does not have a financial relationship with a

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