C-C Chemokine Receptor 2 and Sarcoidosis

Association with Löfgren's Syndrome

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Sarcoidosis is thought to result from the interaction between an unknown environmental antigenic trigger and the host's genetic susceptibility. We hypothesized that sarcoidosis, or one of the disease subsets, could be associated with single nucleotide polymorphisms of C-C chemokine receptor 2 (CCR2) gene. Eight single-nucleotide polymorphisms in CCR2 were studied in a total of 304 Dutch individuals (90 non-Löfgren sarcoidosis, 47 Löfgren's syndrome, 167 control subjects). From the investigated CCR2 polymorphisms, nine haplotypes were deduced (haplotypes 1–9). In patients with Löfgren's syndrome, a strongly significant increase in the frequency of CCR2-haplotype 2, which includes four unique alleles (A at nucleotide position –6752, A at 3,000, T at 3,547, and T at 4,385), was observed compared with control subjects (74% vs. 38% respectively, $p < 0.0001$), whereas no difference was found between non-Löfgren sarcoidosis and control subjects (both 38%). The association between CCR2-haplotype 2 carriage frequency and Löfgren's syndrome (odds ratio, 4.4; $p < 0.0001$) remained significant after adjustment for human leukocyte antigen haplotype DRB1*0301-DQB1*0201 (odds ratio, 11.5; $p < 0.0001$) and female sex (odds ratio, 3.2; $p = 0.003$), two known risk factors for Löfgren's syndrome. In conclusion, this report describes a strong association between CCR2-haplotype 2 and Löfgren's syndrome. Further studies are needed to understand the molecular mechanisms underlying this association.

Keywords: polymorphisms; cytokines; sarcoidosis

Sarcoidosis is a multisystem disease of unknown origin that is characterized by the accumulation of mononuclear cells at disease sites resulting in granuloma formation. The disease is thought to be triggered by unknown environmental antigens in genetically predisposed hosts (1–8). Clinical onset and progression vary widely in sarcoidosis, ranging from benign, self-limited, and often asymptomatic disease to progressive pulmonary fibrosis leading to respiratory failure. A subset of patients has Löfgren's syndrome, which is characterized by the acute presentation of fever, erythema nodosum, bilateral hilar lymphadenopathy, and polyarthralgia (9).

Familial clustering in sarcoidosis (10, 11) has prompted a search for relevant genes in the human leukocyte antigen (HLA) region, an area that is key to the adaptive immune response (12, 13). Although early studies showed associations with class I alleles, it is the HLA class II alleles that have been most frequently reported to be associated with the presence of disease (14). Furthermore, genetic factors may influence disease presentation and progression. The HLA-A1, B8, DR3 haplotype, for instance, is associated with disease of acute onset and short duration (15). Fine mapping along this haplotype has shown DQB1*0201 to be associated with stage I disease and Löfgren's syndrome and to be strongly protective against the progression of pulmonary disease in Dutch and United Kingdom sarcoidosis patients (15, 16).

Genetic determinants of sarcoidosis are likely to reside in loci that influence T-cell function, regulation of antigen recognition and processing, and chemokines and chemokine receptors involved in the recruitment of lymphocytes and mononuclear cells. The C-C chemokine receptor 2 (CCR2), one of the main receptors for monocyte chemoattractant proteins (1–5), plays an important role in recruiting monocytes (17, 18), memory T cells (19), natural killer cells (20, 21), and immature dendritic cells (22), making it a good candidate. A single nucleotide polymorphism (SNP) in the CCR2 gene (G190A), resulting in a conservative amino acid substitution (V64I), has been associated with a lower prevalence of sarcoidosis in a Japanese population (23), with a similar but not significant trend seen in a Czech population (24).

Since the publication of these studies, further SNPs in the CCR2 gene have been reported in publicly available SNP databases, enabling us to fine map this region to determine the primary site(s) for association. Our hypothesis was that genetic variation in CCR2 could affect the interplay between CCR2 and its ligands and thus affect ligand expression and/or the recruitment of mononuclear cells, which play a key role in inflammatory diseases such as Löfgren's syndrome.

METHODS

Dutch Patients and Control Subjects

One hundred thirty-seven unrelated Dutch white sarcoid patients were included in the study. In ninety patients, the diagnosis of sarcoidosis was histologic, with exclusion of other causes of granulomatosis. In the forty-seven patients with classic Löfgren's syndrome, the diagnosis was nonhistologic. Verbal and written patient consents were obtained from all subjects, and authorization was given by the Ethics Committee of the Sint Antonius Hospital, Nieuwegein (Utrecht region).

The Dutch control group was comprised of 167 white healthy donors from the Blood Transfusion Service in Utrecht, which takes donors mainly from the Utrecht region. All donors were routinely checked for health before donation and gave their written consent.

Sequence-specific Primers and Polymerase Chain Reaction

Polymorphisms were determined using sequence-specific primers and polymerase chain reaction that uses sequence-specific primers with 3′-end mismatches and identifies the presence of specific allelic variants through polymerase chain reaction amplification. All polymerase chain reactions were run under identical conditions as previously described (25). The presence of an allele-specific band of the expected size, in conjunction with a control band, was considered positive evidence for each particular allele. The absence of an allele-specific band and the presence of a control band were considered evidence for the absence of an allele.

A total of eight biallelic SNPs were identified in the CCR2 gene at

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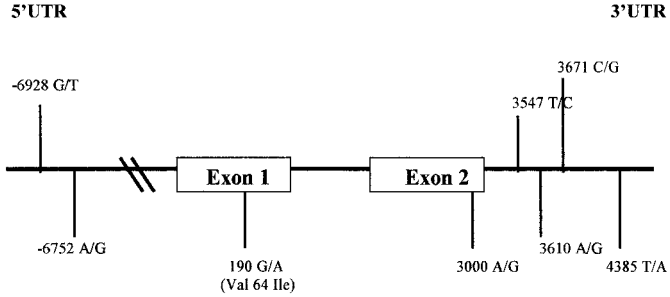

Figure 1. Positioning of the investigated single nucleotide polymorphisms across the C-C chemokine receptor 2 (CCR2) gene.

the following nucleotide positions: −6,928 (G/T; promoter), −6,752 (A/G; promoter), 190 (G/A; exon: Val/Ile), 3,000 (A/G; exon), 3,547 (T/C; 3′UTR), 3,610 (A/G; 3′UTR), 3,671 (C/G; 3′UTR), 4,385 (T/A; 3′UTR). Figure 1 shows a graphic representation of the relative SNP positions in the CCR2 gene. The sequences of the primers that detected these polymorphisms are shown in Table 1.

Analysis of HLA-DRB1 and HLA-DQB1 Genetic Polymorphisms

Genomic DNA from all subjects, extracted from peripheral blood cells, was genotyped for HLA-DRB1 and HLA-DQB1 as previously described (25).

Data Analysis

The genotype frequencies, allele frequencies, and the allele carriage frequencies (i.e., number of individuals carrying the allele either in both [homozygous] or in only one [heterozygous] chromosome) were determined by direct counting. All genotype frequencies were tested for Hardy-Weinberg equilibrium. Haplotypes were identified using the estimate haplotype frequencies program ARLEQUIN (http://lgb.unige.ch/arlequin/), a (widely) accepted method for haplotype estimation (26, 27). Subsequently, the carrier frequency of each haplotype was determined by direct counting. Linkage disequilibrium between individual SNPs was also calculated by using ARLEQUIN software. Proportions were compared using chi-square statistics or Fisher's exact test as appropriate. Adjustment for multiple tests was made using the formula $p_c = p \times n$, where pc is the corrected value, p the uncorrected value, and n the number of tests performed (Bonferroni method). A value of $p < 0.05$ was considered significant. Multivariate polytomous logistic regression was used to assess the independent association between CCR2 haplotypes and disease subsets, while adjusting for age at presentation, sex, and smoking history. To evaluate the interaction between CCR2 and HLA alleles, the likelihood ratio test was used to compare the maximum likelihood models with and without the interaction term between CCR2 and DRB1*0301-DQB1*0201. Statistical analyses were performed using the program STATA 7 (Stata Corp., College Station, TX).

RESULTS

The allele frequencies of the investigated CCR2 polymorphisms for sarcoidosis, Löfgren's syndrome, and control subjects are summarized in Table 2. For each polymorphism, the proportion of heterozygote and homozygote alleles was consistent with Hardy-Weinberg equilibrium (i.e., the observed number of alleles did not significantly differ from the expected frequency, as determined by using chi-squared analysis). In the sarcoidosis group as a whole, increases in the CCR2 −6752A (promoter), 3000A (exon), 3547T (3′UTR), 3610A (3′UTR), 4385T (3′UTR) alleles did not reach statistical significance after correcting for the number of comparisons performed. However, when Löfgren patients were analyzed separately, a strongly significant association with the previously mentioned polymorphisms was observed (Table 2). The other investigated CCR2 polymorphisms, at nucleotide positions 3,671 (C/G, 3′UTR), 190 (G/A, exon coding sequence), −6,928 (G/T, promoter), revealed no differences in genotype, phenotype, and allele frequency between Dutch patients and control subjects.

From the investigated CCR2 polymorphisms, we were able to deduce nine estimated haplotypes (Table 3). Haplotype 2 includes four SNPs (T at nucleotide position 4,385, T at 3,547, A at 3,000, and A at −6,752), which appear to be in 100% linkage, as they occur together in all subjects; these four SNPs were unique to this haplotype alone, being absent in all of the other haplotypes. Univariate polytomous logistic regression was used to compare haplotypes between Löfgren, non-Löfgren sarcoidosis, and control subjects. CCR2-haplotype 2 was significantly overrepresented in the Löfgren's group compared with both control subjects (allele carriage, 74% vs. 38%, respectively; odds ratio [OR], 4.81; 95% confidence interval [CI], 2.3–10.0; $p < 0.0001$) and to the non-Löfgren sarcoidosis group (allele carriage 74% vs. 38%; OR, 4.80; 95% CI, 2.2–10.5; $p < 0.0001$), whereas no difference was found between non-Löfgren sarcoidosis and control subjects (both 38%) (Table 3). After correcting for multi-

TABLE 1. SEQUENCES OF PRIMERS USED FOR PCR-SSP DETECTION OF CCR2 POLYMORPHISMS

SNP's position	Locus	Sequence Accession no	Annealing position	Primer sequence	Control primers*	Product size, bp
−6928 G/T	Promoter	U95626	39151-177	F-TGC ATA AAG AAT TCT AAG ATG TAC TG/T	210 + 211	799
			39930-950	R-AGG GTG AAC AAA GGT TCA CCA		
−6752 A/G	Promoter	U95626	39353-373	R-AAC CAG ATG GGA AGA GGG AAT/C	210 + 211	772
			38602-623	F-GGA AAT TGC TAA GGG CAT CGT T		
190 G/A	Exon coding	U95626	46295-321	R-TTT TTG CAG TTT ATT AAG ATG AGG AC/T	210 + 211	808
			45513-534	F-GAA GGC AGA AGG TGA ATA GTT C		
3000 A/G	Exon	U95626	49083-105	F-CCA GTG GGA ACT CCT AAA TCA AA/G	210 + 211	918
			49981-50000	R-ATA GGT AGA CCC TCC GGG AT		
3547 T/C	3′UTR	U95626	49633-652	F-TAC AGG CCA CAC AAC CCC AT/C	210 + 211	851
			50464-483	R-CCT GCT TAA CTC GAA CAG CC		
3610 A/G	3′UTR	U95626	49695-715	F-GAC ATC TGC CTC ATC CAA GCA/G	210 + 211	867
			50541-561	R-TAT GCC AAG ACC CTT CCT TAC		
3671 C/G	3′UTR	U95626	49776-796	R-CTG TGT CTT CTC ATT CAC CAG/C	210 + 211	870
			48927-946	F-TCG CTG TCA TCT CAG CTG GA		
4385 T/A	3′UTR	U95626	50470-490	F-TCG AGT TAA GCA GGT GGA AGT/A	210 + 211	766
			51214-235	R-AAG GCC CCA TTG AAA CAA TGA C		

* Control primer 210: F-ATGATGTTGACCTTTCCAGGG
Control primer 211: R-TTCTGTAACTTTTCATCAGTTGC

TABLE 2. C-C CHEMOKINE RECEPTOR POLYMORPHISMS ALLELE FREQUENCIES IN LÖFGREN'S SYNDROME, NON-LÖFGREN SARCOIDOSIS, AND CONTROL SUBJECTS

SNPs Position	Allele	Löfgren (*n = 47*)	Non–Löfgren Sarcoidosis (*n = 90*)	Sarcoid Total (*n = 137*)	Control Subjects (*n = 167*)
−6,928	T	6 (0.06)	21 (0.12)	27 (0.09)	28 (0.08)
	G	88 (0.94)	159 (0.88)	247 (0.91)	306 (0.92)
−6,752	A	41 (0.44)*†	42 (0.23)	83 (0.30)	71 (0.21)
	G	53 (0.56)	138 (0.77)	191 (0.70)	263 (0.79)
190	A	5 (0.05)	12 (0.07)	17 (0.06)	21 (0.06)
	G	89 (0.95)	168 (0.93)	257 (0.94)	313 (0.94)
3,000	A	41 (0.44)*†	42 (0.23)	83 (0.30)	71 (0.21)
	G	53 (0.56)	138 (0.77)	191 (0.70)	263 (0.79)
3,547	T	41 (0.44)*†	42 (0.23)	83 (0.30)	71 (0.21)
	C	53 (0.56)	138 (0.77)	191 (0.70)	263 (0.79)
3,610	A	47 (0.50)‡§	56 (0.31)	103 (0.38)	95 (0.28)
	G	47 (0.50)	124 (0.69)	171 (0.62)	239 (0.72)
3,671	G	13 (0.14)	24 (0.13)	37 (0.14)	53 (0.16)
	C	81 (0.86)	156 (0.87)	237 (0.86)	281 (0.84)
4,385	T	41 (0.44)*†	42 (0.23)	83 (0.30)	71 (0.21)
	A	53 (0.56)	138 (0.77)	191 (0.70)	263 (0.79)

Definition of abbreviation: SNP = single nucleotide polymorphism.
Data are given as absolute numbers with percentages in parentheses.
* Corrected p = 0.008, Löfgren vs. non-Löfgren sarcoidosis, chi-square test.
† Corrected p = 0.0008, Löfgren vs. control subjects, chi-square test.
‡ Corrected p = 0.02, Löfgren vs. non-Löfgren sarcoidosis, chi-square test.
§ Corrected p = 0.002, Löfgren vs. control subjects, chi-square test.

ple comparisons, none of the other CCR2 haplotypes were found to be significantly different between the three groups.

On univariate analysis, a markedly increased carriage frequency of the HLA haplotype DRB1*0301-DQB1*0201 (hence abbreviated as DQB1*0201) was found in the Löfgren group (81%) compared with both control subjects (26%; OR, 11.4; 95% CI, 5.1–25.6; $p < 0.0001$) and to non-Löfgren sarcoidosis (17%; OR, 19.5; 95% CI, 7.9–48.3; $p < 0.0001$), whereas no significant difference was found between non-Löfgren sarcoidosis and control subjects. Female sex was also associated with Löfgren's syndrome (OR, 3.1; 95% CI, 1.6–6; p = 0.001).

Multivariate logistic regression analysis revealed that the association between CCR2-haplotype 2 carriage frequency and Löfgren's syndrome (OR, 4.4; 95% CI, 1.9–9.7; $p < 0.0001$) remained significant after adjustment for DQB1*0201 (OR, 11.5; 95% CI, 4.9–26.8; $p < 0.0001$) and female sex (OR 3.2; 95% CI, 1.5–7.0; p = 0.003). Age at diagnosis and smoking history were not significantly associated with Löfgren's syndrome.

When the frequency of CCR2-haplotype 2 was analyzed separately according to the carriage of DQB1*0201, CCR2-haplotype 2 was strongly associated with Löfgren's syndrome in DQB1*0201 positive (OR, 8.02; 95% CI, 2.9–22.3; $p < 0.0001$) but not significantly so in DQB1*0201 negative patients (OR, 1.3; 95% CI, 0.3–5.0; p = NS) (Table 4). To verify whether the effect of the CCR2 haplotype was dependent on carriage of DQB1*0201 and thus whether there was a significant interaction between the two genes, we compared a logistic regression model in which DQB1*0201, CCR2, and sex were entered as separate covariates to one in which an interaction term between DQB1*0201 and CCR2-haplotype 2 was added. The likelihood ratio test comparing the two models failed to show a significant improvement of the model containing the interaction term over the restricted model (likelihood ratio = 3.4, p = 0.1), indicating that the expanded model containing the interaction term does not add significantly to the predictive ability of the model without the interaction term. The effect of the interaction term itself was

TABLE 3. C-C CHEMOKINE RECEPTOR 2 HAPLOTYPE CARRIAGE FREQUENCIES IN LÖFGREN'S SYNDROME, NON-LÖFGREN SARCOIDOSIS, AND CONTROL SUBJECTS

	SNPs Position										
Haplotype	−6,928	−6,752	190	3,000	3,547	3,610	3,671	4,385	Löfgren (*n = 47*)	Non-Löfgren Sarcoidosis (*n = 90*)	Control Subjects (*n = 167*)
1	G	G	G	G	C	G	C	A	27 (57%)	62 (69%)	122 (73%)
2	G	A	G	A	T	A	C	T	35 (74%)*†	34 (38%)	63 (38%)
3	G	G	G	G	C	G	G	A	12 (26%)	18 (20%)	42 (25%)
4	T	G	G	G	C	A	C	A	3 (6%)	10 (11%)	17 (10%)
5	G	G	A	G	C	G	C	A	4 (9%)	9 (10%)	15 (9%)
6	T	G	G	G	C	G	C	A	2 (4%)	6 (7%)	7 (4%)
7	G	G	G	G	C	A	C	A	3 (6%)	2 (2%)	5 (3%)
8	G	G	A	G	C	G	G	A	1 (2%)	3 (3%)	5 (3%)
9	T	G	G	G	C	G	G	A	0	3 (3%)	3 (2%)

Numbers represent haplotype carriage frequencies with percentages in parentheses.
* $p < 0.0001$ Löfgren vs. non-Löfgren sarcoidosis, polytomous logistic regression.
† $p < 0.0001$ Löfgren vs. control subjects, polytomous logistic regression.

TABLE 4. HLA DRB1*0301-DQB1*0201 AND CCR2 HAPLOTYPE 2 ALLELE CARRIAGE IN LÖFGREN'S SYNDROME, NON-LÖFGREN SARCOIDOSIS AND CONTROL GROUP

		Löfgren (n = 47) CCR2 haplotype 2		non-Löfgren Sarcoidosis (n = 90) CCR2 haplotype 2		Controls (n = 167) CCR2 haplotype 2	
		Y	N	Y	N	Y	N
HLA-DRB1*0301-DQB1*0201	Y	31 (82%)	7 (18%)	4 (25%)	12 (75%)	16 (36%)	28 (64%)
Allele carriage	N	4 (44%)	5 (56%)	30 (41%)	44 (59%)	47 (38%)	76 (62%)

also not significant ($p = 0.070$), despite a relatively large OR (4.8; 95% CI, 0.8–26.9). However, the number of patients available for this subanalysis was relatively small (Table 4), and a fairly large type I error could be expected.

DISCUSSION

We report a strong association between CCR2 polymorphisms and Löfgren's syndrome, suggesting a prominent role for this receptor in pathogenesis. The high degree of linkage disequilibrium, extending over a distance of more than 10,000 base pairs across the CCR2 gene, makes it difficult to determine which individual or which combination of SNPs is causative. Although currently a CCR2-haplotype 2 appears to be key, it remains uncertain whether this haplotype, per se, or a single SNP within it, is critical to the development of Löfgren's syndrome. Of the four unique SNPs located within haplotype 2, one is located in the promoter region (−6,752 A/G), another in the exonic noncoding region (3,000 A/G), and two in the 3′ untranslated region (3,547 T/C, 4,385 T/A). Thus, haplotype 2 could influence gene expression through a variety of mechanisms, including differential binding of transcription factors to the promoter polymorphism and alteration of mRNA processing, stability, or translation. Alternatively, haplotype 2 could be a marker of another unidentified polymorphism(s) within CCR2 or of polymorphisms located in neighboring genes. These unanswered points, as well as the relationship of our findings with the report of a weak association between CCR5 and sarcoidosis (24), will form the basis for further investigation.

We have not reproduced the findings of Hizawa and colleagues (23) of a significantly decreased frequency of the CCR2–64I SNP in Japanese sarcoidosis patients (corresponding to our 190A SNP). This disparity might result from ethnic differences. Such differences are apparent in the clinical presentation, as Löfgren's syndrome is extremely rare in Japan.

Our findings have not established whether CCR2-haplotype 2 predisposes to Löfgren's syndrome in all carriers or only in those with DQB1*0201 positivity. In DQB1*0201 positive subjects, CCR2-haplotype 2 carriage was strikingly more prevalent in Löfgren's syndrome (82%) than in control subjects (36%); in contrast, in DQB1*0201-negative subjects, CCR2 carriage differed little between Löfgren's syndrome (44%) and control subjects (38%). However, the small size of the DQB1*0201-negative subgroup in Löfgren's syndrome (n = 9) is an important caveat. Indeed, in view of the fairly large expected type I error caused by the relatively small group of patients available for this secondary analysis, we are not in a position to confidently exclude the possibility of an interaction between DQB1*0201 and CCR2-haplotype 2 carriage. Further studies are needed to determine whether CCR2-haplotype 2 acts as a risk factor that is genetically dependent on the concomitant presence of the DQB1*0201 haplotype or whether it is sufficient on its own.

The association between CCR2-haplotype 2 and Löfgren's syndrome, but not with non-Löfgren sarcoidosis, as well as the presence of an association with DQB1*0201, is intriguing. CCR2 is mainly expressed by monocytes, macrophages, dendritic cells, and T lymphocytes and plays a crucial part in regulating the recruitment of these cells. Mice deficient in CCR2 exhibit defects in monocyte/macrophage/lymphocyte trafficking to sites of inflammation (28). When challenged with high doses of mycobacteria, the CCR2 knockout mice die rapidly of infection, possibly as a result of defective recruitment of macrophages to the lungs, of dendritic cells to the mediastinal lymph nodes, and of interferon-γ producing CD4 and CD8 lymphocytes to both sites (29). A decreased production of interferon-γ in *CCR2*$^{-/-}$ mice infected with *Leishmania donovani* (30) or *Cryptococcus neoformans* (31) has also been reported, suggesting that CCR2-dependent production of interferon-γ is critical for host defense.

The functional consequences of our findings require further definition; however, the CCR2 haplotype associated with Löfgren's syndrome may be linked to the extent and type of monocyte recruitment and subsequently to differences in interferon-γ production. Alternatively, CCR2-haplotype 2 may act as a coreceptor for the putative pathogenic agent(s) of Löfgren's syndrome, which may be distinct from the agent(s) causing non-Löfgren sarcoidosis. In fact, even though the only pathogen hypothesized to bind to CCR2 is human immunodeficiency virus (32), CCR2 may also affect binding to other yet unknown micro-organisms, as has been hypothesized for CCR5 in relationship to a variety of micro-organisms (33, 34).

The existence of at least two clear genetic markers of Löfgren's disease, HLA class II haplotype DRB1*0301-DQB1*0201 and CCR2-haplotype 2, strongly suggests that this disease represents a distinct entity from non-Löfgren sarcoidosis. Further studies are needed to understand the molecular mechanisms that underlie these associations.

Conflict of Interest Statement: P.S. has no declared conflict of interest; E.A.R. has no declared conflict of interest; A.U.W. has no declared conflict of interest; H.S. has no declared conflict of interest; J.C.G. has no declared conflict of interest; P.S. has no declared conflict of interest; A.A. has no declared conflict of interest; E.G. has no declared conflict of interest; H.J.T.R. has no declared conflict of interest; R.M.d.B. has no declared conflict of interest; K.I.W. has no declared conflict of interest.

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