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Association of IBD Risk Loci with Sarcoidosis and its Acute and Chronic Subphenotypes

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Abstract

Sarcoidosis is a complex granulomatous inflammatory disorder that shares several clinical and pathogenetic features with inflammatory bowel disease (IBD). Postulating a common genetic basis of inflammatory diseases, we tested 106 SNPs that are known or have been suggested to be associated with IBD for a potential association with sarcoidosis and its acute and chronic subphenotypes.

We genotyped 1,996 German sarcoidosis patients, comprising 648 acute and 1,161 chronically affected individuals and 2,622 control subjects and 342 German trios with affected offspring using SNPlex™ technology.

The non-synonymous SNP rs11209026 (Arg381Gln) in the *interleukin-23 receptor* (*IL23R*) gene was associated with chronic sarcoidosis ($p = 5.58 \times 10^{-5}$; OR = 0.63), what was supported by the result of a TDT analysis in the independent family sample ($p_{TDT} = 0.031$; OR = 0.50). Marker rs12035082 located at chromosome 1q24.3 was found to be associated with the acute subphenotype ($p = 6.80 \times 10^{-7}$; OR = 1.36) and rs916977 (*HERC2* locus; $p = 4.49 \times 10^{-5}$; OR = 1.30) was associated with sarcoidosis.

Our results highlight the potential importance of the IL23 signaling pathway for the development of chronic sarcoidosis. The finding links sarcoidosis pathogenesis to other inflammatory conditions and may contribute to new hypotheses on disease mechanisms.

Key words: *IL23R*, rs11209026, *HERC2*, rs12035082, inflammation

Introduction

Several immune-related susceptibility genes have been demonstrated as shared risk factors for chronic inflammatory disorders. This includes *PTPN22* and *CTLA4* (various autoimmune diseases [1-2]), *NOD2* (Crohn disease (CD) [3-4]; atopic diseases [5-6]) and *IL23R* (CD [7-8]; psoriasis [9]; ankylosing spondylitis [10-12]). In particular, CD and UC, the two main phenotypes of inflammatory bowel disease (IBD) share a number of susceptibility loci (e.g. *NKX2-3*, *CCNY*, *STAT3*, *PTPN2*) [13-14]. Presumably, this overlap of genetic risk loci as well as familial clustering of different autoimmune diseases [15] indicates that shared biological pathways may be involved in the etiology of clinically distinct immune-mediated diseases (reviewed in [16-18]).

Sarcoidosis (OMIM #181000) is an inflammatory disorder that can be further classified into acute or chronic sarcoidosis according to the course of the disease. It shares several clinical and immunological features with other chronic inflammatory diseases, like general inflammatory characteristics, or more specifically, the formation of non-caseating granuloma in CD. Furthermore, sarcoidosis is a complex polygenic disorder, to which variants in numerous genes and yet unknown environmental factors are likely to contribute [19]. The genetic underpinning in sarcoidosis is supported by the discovery of two susceptibility genes, namely *BTNL2* [20-21] and *ANXA11* [22]. Besides these disease loci, numerous case-control association studies of potential candidate regions have been published for sarcoidosis, but many of them with conflicting results (for review see [23]). This includes the chemokine receptors *CCR2* and *CCR5* of *3p21.3*, *IL18*, *TNF-α* and several HLA loci. Most of these genes seem to play key roles in the pathogenesis of other immune disorders, such as *IL18* or *TNF-α* in CD (for review see [24]). Furthermore, we recently identified a novel shared susceptibility locus on chromosome *10p12.2* for both sarcoidosis and CD by combining genome-wide association datasets from the two diseases [25]. This underlines the aforementioned hypothesis that a number of genetic regions exist that may act as general modifiers of the inflammatory response.

Here, we aimed to identify the putative effects of genetic variants in 74 IBD risk loci on the susceptibility to sarcoidosis and to its acute and chronic subphenotypes. With this approach, we were able to identify *HERC2* as a novel susceptibility gene for sarcoidosis, and *chr12q24.3* and *IL23R* as subphenotype-specific risk loci for the acute and chronic subphenotype.

Materials and Methods

Patients and control subjects

A total of 1,996 unrelated German sarcoidosis patients and 2,622 unrelated German controls were included in the study. All patients were recruited through specialized hospitals and practitioners, the German sarcoidosis patients' organization (Deutsche Sarkoidose-Vereinigung e.V., www.sarkoidose.de), and via health insurance institutions. In the latter case, histological confirmation of the diagnosis was necessary for inclusion of individuals. All patients completed a questionnaire on the course of disease. Patients' physicians were contacted to provide clinical radiology and laboratory data required to confirm the diagnosis of sarcoidosis. According to the clinical presentation of the disease, patients were classified as having either chronic or acute sarcoidosis, if possible. The group of acute sarcoidosis comprised patients with sudden complaints and recovery within two years including patients with Löfgren's syndrome.

Individuals with the chronic phenotype of sarcoidosis exhibited subtly intensifying early symptoms, followed by enduring disease activity for two years or longer. In order to reduce phenotypic heterogeneity, sarcoidosis patients were separated in 648 individuals with acute sarcoidosis, including 105 patients with Löfgren's syndrome, and 1,161 persons with the chronic form of the disease, 734 of which suffered from enduring disease despite cortisone therapy and 427 of which experienced recovery with or without treatment. As the remaining 187 patients could not be classified unequivocally concerning the course of the disease, these individuals were excluded from the subphenotype-specific analysis. German control individuals were recruited from the PopGen biobank [26]. Written informed consent was obtained from all participants. All collection protocols were approved by institutional ethics review boards and data protection authorities of the participating institutes.

The family sample comprised 342 German trios with offspring affected by sarcoidosis. Of these, 121 trios comprised offspring with the acute form of the disease and offspring of 255 trios was affected by chronic sarcoidosis. Recruitment and diagnosis was accomplished as described above for the case-control sample.

SNP Selection, genotyping and quality control

All 106 SNPs included in this study were selected from prior publications for their confirmed or suggested association with CD and/or UC. The majority of these markers (58 SNPs) were originally published by the WTCCC [27] or were identified through the meta-analysis performed by Barrett et al. [14]. See Table S1 in the online data supplement for details. Ligation-based SNPlex™ genotyping [28] was performed as previously described [29]. Genotype assignments were manually confirmed by visual inspection with the Genemapper 4.0 (Applied Biosystems) software. All process data were written to and administered by a in-house database-driven laboratory information management system (LIMS) described previously [30]. All samples and SNPs included in the analysis met the following quality criteria: All samples had a call rate ≥ 0.9; markers had a minor allele frequency ≥ 0.02, a call rate ≥ 0.95 and showed no deviation from Hardy-Weinberg equilibrium (HWE) in the control sample ($p_{HWE} > 0.01$ in the exact test implemented in PLINK v1.06 [31]). For further quality assessment, at total of 129,840 genotypes from 55 randomly chosen SNPs was technically replicated showing a concordance rate of 99.8% between the two batches.

Statistical analysis

Statistical data analysis was carried out using PLINK v1.06 [32]. Allele frequency differences were assessed using a χ^2 test (1df). Nominal P-values were adjusted for multiple testing using Bonferroni correction with a factor of 318, accounting for all 106 markers under study and the three investigated phenotypes. Power calculation for the TDT analysis was conducted using the "Genetic Power Calculator" [33] and for case-control analysis PS power and sample size program [34].

Analysis of specific expression of *IL23R* by RT-PCR

For investigation of tissue and cell-type specific expression patterns of *IL23R*, we used standard RT-PCR procedures on a commercially available human tissue and immune panel from Clontech (Palo Alto, CA, USA). Bronchoalveolar lavage (BAL) cell samples were taken from 5 patients with sarcoidosis and without steroid treatment at the time of BAL and 5 uneffected individuals.

The diagnosis of sarcoidosis was established retrospectively in accordance with previously defined criteria [35] including noncaseating granuloma identified by transbronchial biopsies. All patients and unaffected individuals gave their informed consent to the study. BAL was performed as previously described [36]. The BAL cell suspensions used in this study contained >95% alveolar macrophages. Briefly, the PCR program used was applied as follows: Denaturation for 5 min at 95°C; 33 cycles of 20 sec at 95°C, 20 sec at 53°C, 60 sec at 72°C; final extension for 5 min at 72°C. The following primers were used for amplification: hIL23R-F1: GACACATGGAATTCTGGGCT, hIL23R-R1: CAAAAGCATGGTGGTTTCCT. To confirm the use of equal amounts of RNA in each experiment, all samples were checked in parallel for GAPDH expression using the following primers: GAPDH-F: CCAGCCGAGCCACATCGC, GAPDH-R: ATGAGCCCCAGCCTTCTCCAT. All amplified DNA fragments were analyzed on 1% agarose gels and subsequently documented and quantified on a ChemiDoc XRS system (BioRad, Munich, Germany).

Results

Case-control association analysis

A total of 106 SNPs from 74 different loci passed the quality control criteria (see Methods section). In the association analysis 24 SNPs from 21 different loci reached nominal significance with $p_{nom} < 0.05$ in the allelic χ^2 test (one degree of freedom(df)) in the overall sample of 1,996 sarcoidosis cases and 2,622 controls. In the subphenotype-specific analysis, eight of these SNPs were also nominally associated ($p_{nom} < 0.05$) with acute sarcoidosis (648 cases) and 16 with the chronic form of the disease (1,161 cases). Three distinct SNPs were nominally significant solely for acute sarcoidosis and further four SNPs for chronic sarcoidosis only. Detailed results for all SNPs under study, including genotype counts, are shown in the online data supplement Table S2.

Since every SNP was tested in the overall, in the acute, and in the chronic sample, nominal P-values were Bonferroni-corrected for 3×106=318 independent tests. Three association signals, namely from rs916977, rs11209026 and rs12035082, remained significant after correction for multiple testing (Table 1). SNP rs916977, which is located in intron 12 of the *hect domain and RLD 2 (HERC2)* gene on chromosome 15q13.1 yielded a corrected p-value (p_{corr}) of 0.014 in the overall sarcoidosis sample (OR = 1.30; 95% confidence interval (95% CI) [1.15-1.48]), with both subphenotypes contributing to the signal to a similar extent (OR_{acute} = 1.34; 95% CI [1.12-1.61]; $OR_{chronic}$ = 1.29; 95% CI [1.11-1.50]). These ORs were in the same range as for CD (OR = 1.26; CI [1.07–1.47]), but reduced compared to UC (OR = 1.46; CI [1.23-1.75]), as reported previously for a German sample [37]. Furthermore, two subphenotype-specific associations were observed: The non-synonymous SNP rs11209026 in the *interleukin-23 receptor* (*IL23R*) gene, which is a well established risk factor for CD [7, 14, 37-38], was solely associated with chronic sarcoidosis ($p_{corr,chronic}$ = 0.018; $OR_{chronic}$ = 0.63; 95% CI [0.50-0.79]). The effect of this SNP only slightly differed between patients with enduring disease despite cortisone treatment and patients that experienced recovery (OR = 0.66; 95% CI [0.50-0.68] and OR = 0.57; 95% CI [0.40-0.82], respectively). As IL23R directly interacts with the Janus Kinase 2 (JAK2) on the molecular level [39], it is interesting to note that the two SNPs rs10758669 and rs10974944, located in the *JAK2* gene, are associated with chronic sarcoidosis with nominal significance ($p_{chronic}$ = 0.013; $OR_{chronic}$ = 1.14; CI [1.03-1.26] and $p_{chronic}$ = 0.0061; $OR_{chronic}$ = 1.16; CI [1.04-1.30], respectively). However, no epistatic effects were detected

between *IL23R* and *JAK2* SNPs (data not shown). Finally, an exclusive association with acute sarcoidosis was observed for SNP rs12035082 ($p_{corr,acute} = 2.34x10^{-4}$; $OR_{acute} = 1.36$; CI [1.21-1.54]), which is located downstream of the *tumor necrosis factor (ligand) superfamily, member 18* (*TNFSF18, GITRL*) on chromosome 1q24.3 and was reported to modestly increase the risk for CD (OR = 1.20; CI [1.05-1.37]) [37]. In the "Löfgren" subsample this SNP was not significantly associated, probably due to the small number of patients (n = 105; power = 0.34).

Additional evaluation in a family-based design

In order to substantiate these novel associations, we tested the three markers in an independent family sample comprising 342 German trios with affected offspring, including 121 "acute" trios and 255 "chronic" trios, using the transmission disequilibrium test (TDT; Table 2). SNP rs916977 located in the *HERC2* gene, which was found to be associated with sarcoidosis in the population-based design (OR = 1.30), yielded a borderline, but not significant result (p = 0.069; OR = 0.73; 95% CI [0.52-1.03]; 29% power). Marker rs11209026, located in the *IL23R* gene, showed a nominal significant association with the chronic subphenotype ($p_{chronic} = 0.031$; OR = 0.50; 95% CI [0.26-0.95]) and a similar effect size as in the case-control analysis (OR = 0.63). Finally, SNP rs12035082 was found to have yielded a slightly higher OR (OR = 1.50; 95% CI [0.96-2.35]) in the acute trios than in the population-based design (OR = 1.36), but also only a borderline non-significant p value ($p_{acute} = 0.074$; 27% power).

Expression analysis of *IL23R*

To gain first insights on a possible effect of *IL23R* variants regarding sarcoidosis, the transcript levels of *IL23R* were assessed by RT-PCR in a panel of different healthy human tissues and resting and activated immune cells (Fig. 1). The expression analysis in this panel showed elevated *IL23R* expression in testis and activated CD4+ T cells and moderate expression in healthy lung tissue. Next, we analyzed the expression levels of *IL23R* in cells derived from bronchoalveolar lavage (BAL) cells using quantitative real-time PCR and cDNA from sarcoidosis patients and unaffected controls (n = 5 per group). Statistical analysis revealed no significant differences in relative expression levels between patients and controls (data not shown).

Discussion

Under the hypothesis of a common genetic basis of inflammatory diseases, we selected SNPs that are known to be associated with CD or UC as likely candidates for also playing a role in sarcoidosis etiology. We identified three significant associations with sarcoidosis with two being specific to the acute and chronic subphenotypes.

The overall sarcoidosis phenotype was associated with marker rs916977, which is a non-synonymous SNP in the *HERC2* gene. Our study sample originated from Germany and therefore we do not expect a significant population structure for this sample [40]. However, allele frequencies of markers located at the *HERC2* locus have recently been shown to exhibit a North-South gradient across Europe [41], possibly even within Germany. *HERC2* was previously reported to be associated with CD and UC in a German and in a Dutch-Belgian sample [37-38]. However, neither the association to CD and UC nor to sarcoidosis was investigated in a well-powered family-based study design and to date there are no reports on a possible functional role

of HERC2 in the inflammatory process. The association signal for *HERC2* has therefore to be considered with caution and requires further investigation.

The association of *IL23R* variants with IBD and with a number of chronic inflammatory diseases is well established [7, 9-13, 42-46]. Our study is the first report on an association of an *IL23R* variant with chronic sarcoidosis (rs11209026; OR = 0.63; 95% CI [0.50-0.79]) and provides further support for this signal by the nominal significant association in an independent family sample (OR = 0.50; 95% CI [0.26-0.95]). The associated SNP rs11209026 (Arg381Gln) causes an amino acid exchange in the intracellular domain of the IL23 receptor and may thus affect downstream signaling of the receptor. Preliminary expression studies showed elevated *IL23R* expression in activated CD4+ T cells and low expression in monocytes what is in line with previous findings [39]. In addition, we found moderate expression in healthy lung tissue. Differential *IL23R* expression has been reported for CD4+ T cells from UC and CD patients [47]. However, the lack of differential expression of *IL23R* in BAL cells from sarcoidosis patients compared to healthy individuals (data not shown) does not contradict our genetic finding mainly because the causative SNP(s) may influence IL23R function rather than mRNA expression.

Signal transduction through the IL23 receptor and the downstream elements of the IL23 pathway is well characterized. IL23 signaling is crucial for the induction of Th17 cells [48], and there is increasing evidence that this is a key pathway in the development of chronic inflammation in the course of various skin, intestinal and lung inflammatory diseases (reviewed in [49-52]). However, to date the particular role of Th17 cells in the pathogenesis of sarcoidosis has not been investigated [53]. The tyrosine kinase JAK2 is directly involved in signal transduction from the functional receptor complex consisting of IL23R and IL12RB1. The nominally significant genetic association of chronic sarcoidosis with rs10974944, which is located in the *JAK2* gene (OR = 1.17; CI [1.05-1.30]) could therefore provide an additional hint to the potential importance of IL23 signaling for the development of this phenotype, although no formal genetic SNP-SNP interaction was detected. Future experiments will clarify the role of associated SNP rs11209026 and possibly other causative variants in the development of chronic sarcoidosis, and how the genetic variation influences IL23R function regarding sarcoidosis pathogenesis.

SNP rs12035082 located on chromosome 1q24.3 was significantly associated with acute sarcoidosis in the population-based design. Replication in the independent German family sample failed most likely due to low power (27%), while the OR in the TDT analysis points to a true finding. The association signal cannot easily be attributed to a specific gene with potential functional relevance since the SNP is located approximately 112 Mb from the next gene. No conclusions could be drawn from in-house genome-wide association data [22], whether the signal extends further to the *TNFSF18 (GITRL)* or even further to the *TNFSF4 (OX40L)* gene (data not shown). Both have been shown to regulate T cell responses [54-55], reviewed in [56-57], they could both be plausible candidates for genes with functional relevance in sarcoidosis etiology. In order to assess an association of this region and the suggested candidate genes, validation in an independent population and fine-mapping experiments are necessary.

In general, only limited conclusions can be drawn from negative findings in this variant-centered, candidate gene approach, where we tested previously reported IBD risk variants. Disregarding the power issue, there are two major reasons, why a negative finding does not exclude the specific locus as a sarcoidosis risk gene: First, for distinct diseases “private” causative variants may exist at a common risk locus, what results in the observed overlapping pathology, e.g.

granuloma formation. Second, it must be assumed that the majority of the investigated markers here do not represent causative SNPs, but are merely in allelic association with them. Therefore different LD structures between study populations can lead to false negative results. For example, SNP rs2066844 located in the CD risk locus *NOD2* was very recently reported to be associated with a severe course of sarcoidosis [58]. Two CD risk variants (rs10521209 and rs2076756) in the *NOD2* gene region were included in our study, but were not associated with either sarcoidosis or the clinical subphenotypes in this study.

In summary, this study identified the association of *IL23R* variant Arg381Gln with chronic sarcoidosis and provides support for this novel finding by TDT analysis in an independent family sample. We further report on an association of rs916977 (*HERC2*) with overall sarcoidosis and of rs12035082 (chr1q24.3 locus) with the acute subphenotype. This genetic result may point to possible etiological similarities between sarcoidosis and other chronic inflammatory diseases, such as IBD, psoriasis and ankylosing spondylitis [7, 10, 18, 43]. Our study adds further evidence to the postulated common genetic basis of inflammatory disorders. The finding may lead to new hypotheses on disease pathogenesis and encourages further functional investigation of potential causative *IL23R* variants and their effect on the IL23 signaling pathway in the context of sarcoidosis.

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Table 1: Results for the three SNPs that remained significantly associated with overall (SA), acute (AC) or chronic (CH) sarcoidosis after Bonferroni correction for multiple testing.

dbSNP	Locus	Chr	Position	A1	A2	MAF_{UNAFF}	MAF_{SA}	OR_{SA} [95% CI]	p_{SA}	MAF_{AC}	OR_{AC} [95% CI]	p_{AC}	MAF_{CH}	OR_{CH} [95% CI]	p_{CH}
rs11209026	*IL23R*	1	67,478,546	A	G	0.07	0.05	0.71 [0.59-0.85]	6.71E-02	0.06	0.84 [0.64-1.09]	1	0.04	0.63 [0.50-0.79]	**1.79E-02**
rs12035082	*1q24.3*	1	171,165,000	C	T	0.39	0.42	1.15 [1.06-1.25]	3.73E-01	0.46	1.36 [1.21-1.54]	**2.34E-04**	0.40	1.07 [0.97-1.18]	1
rs916977	*HERC2*	15	26,186,959	A	G	0.11	0.13	1.30 [1.15-1.48]	**1.44E-02**	0.14	1.35 [1.13-1.62]	3.49E-01	0.13	1.29 [1.11-1.50]	1

Bold: significant results. Abbreviations: chromosome (Chr), nucleotide of allele 1 (A1) and 2 (A2), frequency of the minor allele (MAF) for cases (SA=overall SA, AC=acute SA, CH=chronic SA) and controls (UNAFF), odds ratio (OR) with 95% confidence interval (95% CI) and Bonferroni corrected p value (p).

Table 2: Results of the TDT analysis for the three SNPs that were significantly associated in the case-control association analysis.

dbSNP	Locus	Chr	Position	A1	A2	T_{SA}	U_{SA}	OR_{SA} [95% CI]	p_{SA}	T_{AC}	U_{AC}	OR_{AC} [95% CI]	p_{AC}	T_{CH}	U_{CH}	OR_{CH} [95% CI]	p_{CH}
rs11209026	*IL23R*	1	67,478,546	A	G	28	44	0.64 [0.40-1.02]	5.94E-02	10	11	0.91 [0.39-2.14]	8.27E-01	14	28	0.50 [0.26-0.95]	**3.08E-02**
rs12035082	*1q24.3*	1	171,165,000	C	T	155	141	1.10 [0.88-1.38]	4.16E-01	48	32	1.50 [0.96-2.35]	7.36E-02	83	88	0.94 [0.70-1.27]	7.02E-01
rs916977	*HERC2*	15	26,186,959	T	C	56	77	0.73 [0.52-1.03]	6.86E-02	18	18	1.00 [0.52-1.92]	1	33	50	0.66 [0.43-1.02]	6.20E-02

Bold: significant results. Abbreviations: chromosome (Chr), nucleotide of allele 1 (A1) and 2 (A2), numbers of transmitted (T) and untransmitted (U) alleles to the offspring affected with sarcoidosis (SA), acute (AC) and chronic (CH) sarcoidosis; odds ratio (OR) with 95% confidence interval (95% CI) and uncorrected p values (p).

Legend Figure 1: Relative expression of *IL23R* mRNA in healthy tissue (left) and in the immune cell panel (right).