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Genetic association of a gain of function interferon gamma receptor 1 (IFNGR1) polymorphism and the intergenic region LNCAROD/DKK1 with Behçet's disease

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Abstract

Objective: Behçet's disease is a complex systemic inflammatory vasculitis of incompletely understood etiology. We performed a large genetic study in Behçet's disease in a diverse multi-ethnic population.

Methods: A total of 9,444 patients and controls from seven different populations were included in this study. Genotyping was performed using the Infinium ImmunoArray-24 V.1.0 or V.2.0 BeadChip. Analysis of expression data from stimulated monocytes, and epigenetic and chromatin interaction analyses were performed.

Results: We identified two novel genetic susceptibility loci for Behçet's disease, including a risk locus in *IFNGR1* (rs4896243, p value= 2.42×10^{-9}; OR=1.25) and within the intergenic region *LNCAROD/DKK1* (rs1660760, p value= 2.75×10^{-8}; OR= 0.78). The risk variants in *IFNGR1* significantly increase *IFNGR1* mRNA expression in lipopolysaccharide-stimulated monocytes. In addition, our results replicated the association (p value< 5×10^{-8}) of six previously identified susceptibility loci in Behçet's disease: *IL10*, *IL23R*, *IL12A-AS1*, *CCR3*, *ADO*, and *LACC1*, reinforcing these loci as strong genetic factors in Behçet's disease shared across ancestries. We also identified >30 genetic susceptibility loci with a suggestive level of association (p value< 5×10^{-5}), which will require replication. Finally, functional annotation of genetic susceptibility loci in Behçet's disease uncovered their possible regulatory roles and suggested potential causal genes and molecular mechanisms that could be further investigated.

Conclusion: We performed the largest genetic association study in Behçet's disease to date and revealed novel putative functional variants associated with the disease. We also replicate and extend the genetic associations in other loci across multiple ancestries.

Keywords

Behçet's disease; vasculitis; IFNGR1; eQTL

Introduction

Behçet's disease is a chronic relapsing-remitting inflammatory disorder characterized by recurrent oral and genital ulcers. It is a systemic vasculitis that can affect the eyes, skin, blood vessels, central nervous system, and gastrointestinal tract (1). Behçet's disease is also known as the "Silk Road disease" as its highest prevalence coincides with this ancient route, stretching from Japan to the Mediterranean region (2). However, patients with Behçet's disease have been diagnosed worldwide (3). Although the etiology and pathogenesis of Behçet's disease remain incompletely understood, it is suspected that environmental factors, such as infectious agents and others, might trigger the onset of the disease in genetically predisposed individuals by propagating a dysregulated immune response (4).

The genetic studies performed to date in Behçet's disease have clearly established the human leukocyte antigen (*HLA)* class I region as the most robust genetic susceptibility locus for the disease (5). Although the association with the classical HLA allele *HLA-B*51* has been replicated in multiple ancestries, several additional loci within the HLA region, including a putative functional variant between *HLA-B* and *MICA*, have been reported (6–8). Outside the HLA region, at least 16 loci have been reported to be associated with Behçet's disease at a genome-wide level of significance (9–16). These genetic susceptibility loci, such as *IL10*, *IL23R-IL12RB2*, *STAT4*, and *FUT2*, among others provided important insights into the pathogenic mechanisms that could be underlying the pathophysiology and immune dysregulation in Behçet's disease.

Despite the progress in understanding the genetic etiology of Behçet's disease, the majority of genome-wide association studies to date in this disease reported data predominantly derived from one or two ancestral populations. In addition, currently available studies are limited by a relatively small sample size compared to genetic studies in other immune-mediated diseases, which is in part due to the low prevalence of Behçet's disease in many populations.

We performed a large genetic association study involving 9,444 individuals representing Behçet's disease patients and controls from seven diverse populations around the world. We identified two novel genetic associations in Behçet's disease, most notably including a susceptibility variant that increases the expression of interferon gamma receptor 1 gene in monocytes. In addition, we extended the association of several previously reported genetic susceptibility loci to other populations and identified >30 loci with a suggestive association that provide additional insights into the pathogenesis of Behçet's disease.

Methods

Study population

A total of 9,444 individuals (3,477 patients and 5,967 controls) were included in this study. All patients fulfilled the 1990 International Study Group classification criteria for Behçet's disease (17). Our study population consisted of seven independent cohorts of diverse ancestries: Turkish (1,317 cases and 699 controls), Spanish (278 cases and 1,517 controls), Italian (144 cases and 1,270 controls), Korean (200 cases and 200 controls), Tunisian (136 cases and 186 controls), Japanese (120 cases and 218 controls), and Western European (67 cases and 599 controls). Genotyping was performed using the Illumina Immunochip custom arrays (Infinium ImmunoArray-24 V.1.0 or V.2.0 BeadChip) according to the manufacturer's instructions. Additional genotyping data from 1,215 and 1,278 Turkish cases and controls, respectively, were obtained from dbGAP (accession number (phs000272.v1.p1) (9). A detailed description of the study population can be found in Supplementary Table 1. The study was approved by the institutional review boards and the ethics committees at all participating institutions and all study participants signed an informed written contest.

Data quality assessment and measures

The same stringent quality control (QC) measures were applied separately in each population cohort, to maintain consistency across populations, using PLINK v.1.9 (18). Single nucleotide polymorphisms (SNPs) were removed if they had a genotyping call rate <98%, minor allele frequencies (MAF) <1%, or deviating from Hardy-Weinberg equilibrium in either cases or controls (HWE; p value $< 1 \times 10^{-3}$). SNPs on sex chromosomes were not analyzed. In addition, samples with a genotyping call rate <95% were filtered out. Relatedness was assessed and one individual from each pair of duplicates and/or first-degree relatives (Pi-HAT>0.4) was randomly excluded.

To control for possible population stratification, principal component analysis (PCA) was performed using a set of linkage disequilibrium (LD)-pruned markers, pairwise $r^2<0.20$, with Eigensoft 6.1.4 software (19). Individuals >6 standard deviations from the cluster centroids were considered outliers and were not included in the analyses. Dot plots showing the first two principal components (PC) were generated for each population using R 3.6 software (20) and are shown in Supplementary Figure 1.

Imputation

Post-QC genotyping data were used for the imputation of autosomal SNPs which was conducted for each population independently with the Michigan Imputation Server using Minimac3 (21). The software SHAPEIT (22) was used for haplotype reconstruction with the Haplotype Reference Consortium (HRC) r1.1 (23) as the reference population. Only SNPs with stringent correlation values ($R^2 > 0.9$) were maintained for further analyses. Finally, additional QC were conducted and variants with MAF< 1% or HWE p value $< 1 \times 10^{-3}$ were excluded.

Data analysis

PLINK v.1.9 (18) was used to perform association analyses. First, logistic regression for each population independently was assessed. The five first principal components were used as covariates. Genomic inflation factor (λ) was calculated per cohort using a set of around 3,000 SNPs included in the Immunochip, known as 'null' SNPs, that have not been previously associated with immune-mediated diseases. Quantile-quantile (Q-Q) plots for the p values are shown in Supplementary Figure 2. Then, we performed a multi-ethnic meta-analysis by means of the inverse variance method including the results of the logistic regressions for all populations. Heterogeneity of associations was tested using Cochran's Q test p value (Q) and heterogeneity Index (I^2). A fixed-effects model was applied for those SNPs without evidence of heterogeneity ($Q > 0.1$ and $I^2 < 50\%$). $Q \leq 0.1$ and $I^2 \geq 50\%$ indicate evidence of heterogeneity and a random-effect model was applied in that case. The commonly used genome-wide threshold of $\leq 5 \times 10^{-8}$ was established for significant associations, and the SNP showing the lowest p value within each associated genomic region was reported as the lead SNP. In addition, a threshold of p value $\leq 5 \times 10^{-5}$ was established for suggestive associations. Next, we performed joint conditional analysis using GCTA software to determine if multiple independent associations exist within an associated genomic region (24, 25). This method uses the summary statistic from the meta-analysis and corrects for LD. Genotyping data from the seven populations were used to estimate the LD patterns used as reference and the lead SNP was included as covariate. We considered independent signals if a variant reached a conditional p value $\leq 5 \times 10^{-8}$. Both associated and suggestive genomic regions are named, in figures and tables, by the bounding genes except in the cases in which the literature repeatedly involves a specific gene. Finally, the qqman R package was used to generate the Manhattans and Q-Q plots.

To check if the overall risk allele frequencies identified in our study were different across our study populations, we first obtained the frequencies of the associated and suggestive variants (p value $\leq 5 \times 10^{-5}$) for cases and controls independently using PLINK v.1.9. Only variants that were present in at least six of the seven populations after quality control were considered. One-way ANOVA was performed using GraphPad Prism version 8.1.1 (GraphPad Software, La Jolla California USA) and p values < 0.05 were considered significant. Mean and standard deviation were also obtained.

Functional annotation

To try to better understand the statistical associations in the disease context, we evaluated the potential causalities of the identified associated variants by performing a comprehensive functional annotation. First, we explored RegulomeDB to annotate the SNPs with regulatory elements and get a probability score of how likely each variant plays a regulatory role (26). This score ranges from 0 to 1, with 1 being the most likely to be a regulatory variant. In addition, we queried HaploReg v4.1 for the epigenomic annotations (27) and the webtool Capture Hi-C plotter (CHiCP), https://www.chicp.org/chicp/, to evaluate chromatin interactions between SNPs and gene promoter regions (28). We also interrogated if our associated variants have been described to act as expression quantitative trait loci (eQTL) through the web tool FUMA GWAS (http://fuma.ctglab.nl) and thorugh HaploReg v4.1.

We used expression and genotyping data from a previous study for the representation of the eQTL of *IFNGR1* (29, 30). Briefly, expression profiling of primary CD14+ monocytes after lipopolysaccharide stimulation for 2 hours from 260 European individuals was performed with HumanHT-12v4 BeadChip (Illumina) and genotyping was performed using the HumanOmmiExpress-12v1.0 BeadChip (Illumina) as previously described (29, 30). These data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test.

Finally, we explored the GWAS Catalog (https://www.ebi.ac.uk/gwas) to assess pleiotropic effect of our associated signals.

Results

After filtering with stringent quality controls, we analyzed a total of 8,982 individuals (3,197 patients) from seven different populations: Turkish, Spanish, Italian, Korean, Tunisian, Japanese, and Western European. A summary of sample/variant QC is shown in Supplementary Table 2. Association test was performed within each ancestry using a logistic regression model (Supplementary Figure 3), and genomic control analysis showed no evidence of population stratification for any cohort (genomic inflation factor, $\lambda < 1.04$; Supplementary Table 2). Next, we undertook a multi-ethnic meta-analysis to combine the results of the seven populations (Figure 1). Consistent with our current knowledge of the disease, the strongest association was observed within the HLA region which has been previously characterized (6, 12, 13).

Excluding the well-known *HLA* region, our results revealed 62 variants at genome-wide significance level (p value $< 5 \times 10^{-8}$) that mapped onto eight different genomic regions. Detailed results of all these variants, including the association results for each population independently are shown in Supplementary Table 3. We performed joint conditional analysis to test if more than one variant within the associated genomic regions was independently associated with Behçet's disease. This approach did not identify any additional independent signals for any of the eight loci; conditional p values are shown in Supplementary Table 4. Therefore, we used the strongest associated variant within each locus as the lead SNP of the association. Results of the lead SNP of each locus are illustrated in Table 1. Two of these eight loci (*IFNGR1* and the intergenic region *LNCAROD/DKK1*) are novel genetic associations in Behçet's disease, while the remaining six loci have been previously reported.

Of the two novel associated loci, the most strongly associated signal was located near *IFNGR1*, *interferon gamma receptor 1*, (lead SNP rs4896243, p value= 2.42×10^{-9}; odds ratio (OR)=1.25). This genomic region also harbored two additional genome-wide associated variants (rs4896242, p value= 4.62×10^{-9}, and rs1327474, p value=8.35×10^{-9}) representing the same signal of association. Consistent odds ratio directions were observed across ancestries. These polymorphisms are in high LD, which is also reflected by the results of the conditional analysis Supplementary Table 4. No additional markers in LD were included in the analyses, as is illustrated in the regional plots (regional plots showing the results of this genomic region in each population are shown in Supplementary Figure 4).

In common with most genetic variants associated with immune-mediated diseases, the *IFNGR1* polymorphisms identified in our study reside in non-coding regions. Therefore, we carried out a comprehensive functional annotation to try to decipher the causal mechanisms of this association (Table 2 and Supplementary Table 5). Epigenetic annotation revealed colocalization of the three associated variants with enhancer histone marks. In addition, rs1327474 colocalizes with promoter histone marks and DNase hypersensitivity sites in multiple primary tissues and cell types, including blood cells. Finally, Chip-Seq data revealed that the variant is located in the binding site of POL2 in a B cell line (GM12891). Because these data suggest a potential regulatory role of these polymorphisms in *IFNGR1*, we checked if these variants have been identified to act as eQTL (Table 2 and Supplementary Table 6). Interestingly, a previous study found that the variants rs4896243 and rs1327474 act as eQTL for *IFNGR1* expression in monocytes after a 2-hour lipopolysaccharide (LPS) stimulation (p value= 2.07×10^{-18} and 2.18×10^{-18}, respectively) (29). Our analysis of these data revealed that the Behçet's disease associated risk alleles increased the expression level of *IFNGR1* (Figure 2). Finally, physical chromatin interactions between these polymorphisms and other genes such as *TNFAIP3*, *IL22RA* and *OLIG3* have been detected in different blood cell types (Supplementary Table 7 and Supplementary Figure 5).

We also reported two genome-wide associated SNPs located in an intergenic region between *LNCAROD, lncRNA activating regulator of DKK*, and *DKK1*, *dickkopf WNT signaling pathway inhibitor 1,* (lead SNP rs1660760, p value= 2.75×10^{-8}; OR= 0.78) representing a new associated signal with Behçet's disease. Only genetic data for the Turkish population were maintained in this locus after QC and none of these variants showed high LD with any other genotyped or imputed SNPs (Supplementary Figure 4). The lead SNP, rs1660760, has been reported to act as an eQLT in brain tissue (Table 2 and Supplementary Table 6).

In addition to the two new susceptibility loci we report in this study, we replicate with a genome-wide level of significance six previously described loci in Behçet's disease: *IL10*, *interleukin 10* (lead SNP rs3024490, p value= 2.81×10^{-10}; OR= 1.26); *IL23R*, *interleukin 23 receptor* (lead SNP rs6660226, p value= 1.01×10^{-10}; OR= 0.79); *IL12A-AS1, IL12A antisense RNA1* (lead SNP rs76830965, p value= 3.43×10^{-12}; OR= 1.66); *CCR3, C-C motif chemokine receptor 3* (lead SNP rs2087726, p value= 9.33×10^{-10}; OR= 0.79); *ADO, 2-aminoethanethiol dioxygenase* (lead SNP rs12220700, p value= 3.07×10^{-8}; OR= 0.80); *LACC1, laccase domain containing 1* (lead SNP rs2121034, p value= 9.44×10^{-9}; OR= 0.79). Consistent OR directions were observed across ancestries for each locus. These data reinforce the strength of these associations and provide evidence of a shared genetic background across ancestries in Behçet's disease (Supplementary Table 3).

We further evaluated the possible functional implications of the genetic variants identified in this study that are within the six loci previously identified with a GWAS level of significance in Behçet's disease. We found overlap with epigenetic features for all these six loci (Table 2, Supplementary Table 5 and Supplementary Figure 6). Most of the polymorphisms identified as susceptibility variants for Behçet's disease within these loci may change regulatory motifs and potentially alter transcription factor binding, and a significant proportion of variants overlapped with promoter and/or enhancer histone marks in at least one tissue and/or cell

type. In addition, most variants appear to act as eQTL, thus modifying gene expression levels (Supplementary Table 6). Notably, the disease risk alleles in *IL10* are associated with reduced expression of *IL10* in whole blood. The Behçet's disease associated variants in *IL23R* identified in our meta-analysis are also associated with altered expression levels in whole blood for the following genes: *PHKB* (*phosphorylase kinase regulatory subunit beta*), *BATF2* (*basic leucine zipper ATF-like transcription factor 2*), *CYB5R4* (*cytochrome b5 reductase 4*), *DRR1* (*family with sequence similarity 107 member A*) and *SLC35D1* (*solute carrier family 35 member D1*). Finally, Hi-C data revealed that most of genetic variants associated with Behçet's disease with a GWAS level of significance in our study showed physical chromatin interaction with gene promoter regions (Supplementary Table 7). Of special note are those interactions between SNPs and the promoters of the genes whose expression levels were affected in the same cell type, such as the interactions between the Behçet's disease associated *CCR3*-variants and the promoters of *CCR1* (*C-C motif chemokine receptor 1*) and *CXCR6* (*C-X-C motif chemokine receptor 6*) in immune-cells. These analyses support the idea that additional genes might be involved in the pathology of Behçet's disease and might represent potential targets that could be further investigated.

Our study also revealed evidence for suggestive association (p values $< 5 \times 10^{-5}$) in 752 additional SNPs corresponding to 39 genomic regions (including SNPs within *LACC1*, *CCR3* and *IL23R*) (Supplementary Table 8). Among these loci, it is worth to highlight our findings in genes that have been previously associated with Behçet's disease such as *IL1A-IL1B, interleukin 1 alpha-interleukin 1 beta,* (lead SNP rs35145107, p value= 1.56×10^{-6}); *IRF8, interferon regulatory factor 8* (lead SNP rs6540239, p value= 3.61×10^{-7}); and *UBAC2*, *UBA domain containing 2* (lead SNP rs4771332, p value= 8.38×10^{-6}). Additional suggestive associations in our study include *IRF5, interferon regulatory factor 5* (lead SNP rs192829776, p value= 6.40×10^{-6}) and *LBP*, *lipopolysaccharide binding protein* (lead SNP rs139169382, p value= 4.36×10^{-5}), among others (Supplementary Table 8). Results from our data in genetic variants previously reported to be associated with Behçet's disease with a GWAS level of significance are shown in (Supplementary Table 9).

Discussion

The present study corresponds with the largest genetic association study reported to date in Behçet's disease. Our results identified two novel genetic regions associated with Behçet's disease, a gain of function *IFNGR1* polymorphism and variants in the intergenic region *LNCAROD/DKK1*. In addition, our data replicate and extend across ancestries the association of six previously reported genetic susceptibility loci for this disease.

We have reported for the first time the involvement of *IFNGR1* as a susceptibility locus for Behçet's disease. *IFNGR1* encodes the binding subunit, alpha chain, of the gamma interferon receptor. The binding of interferon gamma, IFN-γ, stimulates the activation of the Janus kinase (JAK) and signal traducer and activator of transcription (STAT) signaling pathway, which is crucial for the activation of the immune system (31). Interestingly, Tulunay and colleagues observed an increase of the JAK/STAT signaling in both CD14+ monocytes (p value = 9.55×10^{-3}) and in CD4+ lymphocytes (p value= 8.13×10^{-4}) in Behçet's disease patients compared with healthy individuals (32). Our functional annotation

analysis strongly suggested a regulatory role of the *IFNGR1* associated variants. Indeed, we demonstrate that the Behçet's disease risk alleles in this locus increase *IFNGR1* expression in monocytes after 2-hours of LPS stimulation. Few studies analyzing the involvement of monocytes in Behçet's disease have been published to date (32–35). Considering that the knowledge of the context and cell types that determine the strength of the eQTLs may help to identify molecular mechanisms relevant to the disease (36), further research focused on elucidating the role this genetic association in monocytes and its effect on Behçet's disease related pathophysiology is warranted. IFNG has been shown to play a key role in multiple molecular processes that are essential for a normal immune response such as promoting macrophage activation, orchestrating activation of the innate immune system, regulating Th1/Th2 balance, enhancing antigen presentation, and mediating antiviral and antibacterial immunity (37, 38). Noteworthy, *IFNGR1* polymorphisms have been associated with susceptibility to several infectious agents including Helicobacter pylori, Mycobacterium tuberculosis, and Hepatitis B virus (39–41). All these evidences support the hypothesis that an infectious agent acts as a trigger for the onset of the Behçet's disease in individuals with predisposing genetic background, and highlight monocytes as a relevant cell type into the pathophysiology of Behçet's disease. In addition, these data support a potential role for JAK/STAT inhibitors as therapeutic consideration for clinical trials in Behçet's disease (42).

Interestingly, an intronic variant in *IFNGR1*, rs7749390, has been recently identified as a genetic factor for mouth ulcers, albeit with a modest effect (OR= 1.08, 95% confidence interval 1.07–1.08) (43). This variant only passed the quality control measures in the Tunisian population in our study which limited the statistical power to detect a genome-wide level association. However, our results showed a nominal association for this SNP with Behçet's disease (OR= 1.53, 95% confidence interval 1.05–2.21, p value= 1.36×10^{-2}). In addition, the *IFNGR1*-associated variants identified in our study and rs7749390 are in LD, suggesting that they might correspond with the same signal (Supplementary Table 10).

The results of our study also revealed a new genetic signal within an intergenic region between the *LNCAROD* and *DKK1*. *LNCARDOD* encodes a long intergenic non-protein coding RNA which act as an activating regulator of DKK1. DKK1 inhibits the beta-catenin-dependent Wnt signaling by binding to the co-receptor LRP6 (44). Wnt signaling has been shown to play a crucial role in several biological processes, including cellular proliferation, angiogenesis, and development of the immune system (45, 46). In addition, recent evidence suggested the pathogenic involvement of DKK1 through the Wnt signaling pathway in immune-mediated diseases such as rheumatoid arthritis, psoriasis, systemic sclerosis, systemic lupus erythematosus, and ankylosing spondylitis (45, 47). Therefore, Wnt signaling has gained an increasing interest as therapeutic targets in immune-mediated diseases (47). However, considering that these polymorphisms have been only identified in the Turkish cohort in our study, replication as well as functional studies are needed.

Our results replicated the association of *IL10*, *IL23R*, *IL12A-AS1, CCR3, ADO*, and *LACC1* in Behçet's disease. In addition, several of the associated variants in these loci have been reported to be associated with other immune-mediated disorders and/or infectious agents which indicates a pleiotropic effect of these genetic variants (Supplementary Table 11). However, the causal mechanisms of these genetic associations remain unclear.

Functional annotation analysis can reveal predicted functional effects and generate testable hypotheses. As an example, associated SNP in *IL10* and *IL12A-AS1* loci colocalize with promoter and enhancer histone marks in multitude of cell types. *IL23R* variants have been identified to modify the expression levels of ten different genes. It is worth to highlight *CCR3*-associated polymorphisms which act as eQTL for *CCR1* and *CXCR6* and show evidence for chromatin interactions the promoters of these genes in blood cells. These predicted functional effects expand the genomic associations to several target genes that could be further investigated to decipher the exact molecular mechanisms involved in the pathophysiology of Behçet's disease.

Finally, we observed significant differences in the risk allele frequencies of the variants identified in our study (p values $< 5 \times 10^{-5}$) across populations for both cases and controls (Supplementary Table 12). Overall, the results suggest that the frequency of genetic variants identified in this study are in line with the prevalence data of Behçet's disease, showing the highest mean frequencies the Tunisian, Turkish and Asian populations (2, 3).

In conclusion, we present the results of a large multi-national collaborative effort and dense genotyping in immune-related genetic loci to understand the genetic basis of Behçet's disease. We identified novel genetic susceptibility loci for the disease, including a genetic association with a gain of function variant in *IFNGR1* and genetic variants in the intergenic region *LNCAROD/DKK1*. We replicated a number of previously identified genetic susceptibility loci for Behçet's disease and extended them across diverse populations and ancestries. In addition, our functional and epigenetic annotation analysis revealed potential new candidate genes involved in Behçet's disease. Furthermore, over 30 additional loci were revealed with a suggestive level of association, and which will require further validation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Bettiol A, Prisco D, Emmi G. Behcet: the syndrome. Rheumatology (Oxford). 2020;59(Supplement_3):iii101–iii7. [PubMed: 32348523]
2. Verity DH, Marr JE, Ohno S, Wallace GR, Stanford MR. Behcet's disease, the Silk Road and HLA-B51: historical and geographical perspectives. Tissue Antigens. 1999;54(3):213–20. [PubMed: 10519357]
3. Kilian NC, Sawalha AH. Behcet's disease in the United States: A single center descriptive and comparative study. Eur J Rheumatol. 2017;4(4):239–44. [PubMed: 29308276]
4. Mumcu G, Direskeneli H. Triggering agents and microbiome as environmental factors on Behcet's syndrome. Intern Emerg Med. 2019;14(5):653–60. [PubMed: 30523495]

5. de Menthon M, Lavalley MP, Maldini C, Guillevin L, Mahr A. HLA-B51/B5 and the risk of Behcet's disease: a systematic review and meta-analysis of case-control genetic association studies. Arthritis Rheum. 2009;61(10):1287–96. [PubMed: 19790126]
6. Hughes T, Coit P, Adler A, Yilmaz V, Aksu K, Duzgun N, et al. Identification of multiple independent susceptibility loci in the HLA region in Behcet's disease. Nat Genet. 2013;45(3):319–24. [PubMed: 23396137]
7. Ombrello MJ, Kirino Y, de Bakker PI, Gul A, Kastner DL, Remmers EF. Behcet disease-associated MHC class I residues implicate antigen binding and regulation of cell-mediated cytotoxicity. Proc Natl Acad Sci U S A. 2014;111(24):8867–72. [PubMed: 24821759]
8. Gensterblum-Miller E, Wu W, Sawalha AH. Novel Transcriptional Activity and Extensive Allelic Imbalance in the Human MHC Region. J Immunol. 2018;200(4):1496–503. [PubMed: 29311362]
9. Remmers EF, Cosan F, Kirino Y, Ombrello MJ, Abaci N, Satorius C, et al. Genome-wide association study identifies variants in the MHC class I, IL10, and IL23R-IL12RB2 regions associated with Behcet's disease. Nat Genet. 2010;42(8):698–702. [PubMed: 20622878]
10. Takeuchi M, Mizuki N, Meguro A, Ombrello MJ, Kirino Y, Satorius C, et al. Dense genotyping of immune-related loci implicates host responses to microbial exposure in Behcet's disease susceptibility. Nat Genet. 2017;49(3):438–43. [PubMed: 28166214]
11. Mizuki N, Meguro A, Ota M, Ohno S, Shiota T, Kawagoe T, et al. Genome-wide association studies identify IL23R-IL12RB2 and IL10 as Behcet's disease susceptibility loci. Nat Genet. 2010;42(8):703–6. [PubMed: 20622879]
12. Ortiz-Fernandez L, Carmona FD, Montes-Cano MA, Garcia-Lozano JR, Conde-Jaldon M, Ortego-Centeno N, et al. Genetic Analysis with the Immunochip Platform in Behcet Disease. Identification of Residues Associated in the HLA Class I Region and New Susceptibility Loci. PLoS One. 2016;11(8):e0161305. [PubMed: 27548383]
13. Kirino Y, Bertsias G, Ishigatsubo Y, Mizuki N, Tugal-Tutkun I, Seyahi E, et al. Genome-wide association analysis identifies new susceptibility loci for Behcet's disease and epistasis between HLA-B*51 and ERAP1. Nat Genet. 2013;45(2):202–7. [PubMed: 23291587]
14. Xavier JM, Shahram F, Sousa I, Davatchi F, Matos M, Abdollahi BS, et al. FUT2: filling the gap between genes and environment in Behcet's disease? Ann Rheum Dis. 2015;74(3):618–24. [PubMed: 24326010]
15. Hou S, Yang Z, Du L, Jiang Z, Shu Q, Chen Y, et al. Identification of a susceptibility locus in STAT4 for Behcet's disease in Han Chinese in a genome-wide association study. Arthritis Rheum. 2012;64(12):4104–13. [PubMed: 23001997]
16. Kappen JH, Medina-Gomez C, van Hagen PM, Stolk L, Estrada K, Rivadeneira F, et al. Genome-wide association study in an admixed case series reveals IL12A as a new candidate in Behcet disease. PLoS One. 2015;10(3):e0119085. [PubMed: 25799145]
17. Criteria for diagnosis of Behcet's disease. International Study Group for Behcet's Disease. Lancet. 1990;335(8697):1078–80. [PubMed: 1970380]
18. Chang CC, Chow CC, Tellier LC, Vattikuti S, Purcell SM, Lee JJ. Second-generation PLINK: rising to the challenge of larger and richer datasets. Gigascience. 2015;4:7. [PubMed: 25722852]
19. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet. 2006;38(8):904–9. [PubMed: 16862161]
20. R Development Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2019.
21. Das S, Forer L, Schonherr S, Sidore C, Locke AE, Kwong A, et al. Next-generation genotype imputation service and methods. Nat Genet. 2016;48(10):1284–7. [PubMed: 27571263]
22. Delaneau O, Zagury JF, Marchini J. Improved whole-chromosome phasing for disease and population genetic studies. Nat Methods. 2013;10(1):5–6. [PubMed: 23269371]
23. McCarthy S, Das S, Kretzschmar W, Delaneau O, Wood AR, Teumer A, et al. A reference panel of 64,976 haplotypes for genotype imputation. Nat Genet. 2016;48(10):1279–83. [PubMed: 27548312]

24. Yang J, Ferreira T, Morris AP, Medland SE, Genetic Investigation of ATC, Replication DIG, et al. Conditional and joint multiple-SNP analysis of GWAS summary statistics identifies additional variants influencing complex traits. Nat Genet. 2012;44(4):369–75, S1–3. [PubMed: 22426310]
25. Yang J, Lee SH, Goddard ME, Visscher PM. GCTA: a tool for genome-wide complex trait analysis. Am J Hum Genet. 2011;88(1):76–82. [PubMed: 21167468]
26. Boyle AP, Hong EL, Hariharan M, Cheng Y, Schaub MA, Kasowski M, et al. Annotation of functional variation in personal genomes using RegulomeDB. Genome Res. 2012;22(9):1790–7. [PubMed: 22955989]
27. Ward LD, Kellis M. HaploReg: a resource for exploring chromatin states, conservation, and regulatory motif alterations within sets of genetically linked variants. Nucleic Acids Res. 2012;40(Database issue):D930–4. [PubMed: 22064851]
28. Schofield EC, Carver T, Achuthan P, Freire-Pritchett P, Spivakov M, Todd JA, et al. CHiCP: a web-based tool for the integrative and interactive visualization of promoter capture Hi-C datasets. Bioinformatics. 2016;32(16):2511–3. [PubMed: 27153610]
29. Fairfax BP, Humburg P, Makino S, Naranbhai V, Wong D, Lau E, et al. Innate immune activity conditions the effect of regulatory variants upon monocyte gene expression. Science. 2014;343(6175):1246949. [PubMed: 24604202]
30. Fairfax BP, Makino S, Radhakrishnan J, Plant K, Leslie S, Dilthey A, et al. Genetics of gene expression in primary immune cells identifies cell type-specific master regulators and roles of HLA alleles. Nat Genet. 2012;44(5):502–10. [PubMed: 22446964]
31. Kotenko SV, Izotova LS, Pollack BP, Mariano TM, Donnelly RJ, Muthukumaran G, et al. Interaction between the components of the interferon gamma receptor complex. J Biol Chem. 1995;270(36):20915–21. [PubMed: 7673114]
32. Tulunay A, Dozmorov MG, Ture-Ozdemir F, Yilmaz V, Eksioglu-Demiralp E, Alibaz-Oner F, et al. Activation of the JAK/STAT pathway in Behcet's disease. Genes Immun. 2015;16(2):170–5. [PubMed: 25410656]
33. Sahin S, Lawrence R, Direskeneli H, Hamuryudan V, Yazici H, Akoglu T. Monocyte activity in Behcet's disease. Br J Rheumatol. 1996;35(5):424–9. [PubMed: 8646431]
34. Neves FS, Carrasco S, Goldenstein-Schainberg C, Goncalves CR, de Mello SB. Neutrophil hyperchemotaxis in Behcet's disease: a possible role for monocytes orchestrating bacterial-induced innate immune responses. Clin Rheumatol. 2009;28(12):1403–10. [PubMed: 19701803]
35. Tong B, Liu X, Xiao J, Su G. Immunopathogenesis of Behcet's Disease. Front Immunol. 2019;10:665. [PubMed: 30984205]
36. Jonkers IH, Wijmenga C. Context-specific effects of genetic variants associated with autoimmune disease. Hum Mol Genet. 2017;26(R2):R185–R92. [PubMed: 28977443]
37. van de Wetering D, de Paus RA, van Dissel JT, van de Vosse E. Functional analysis of naturally occurring amino acid substitutions in human IFN-gammaR1. Mol Immunol. 2010;47(5):1023–30. [PubMed: 20015550]
38. Tau G, Rothman P. Biologic functions of the IFN-gamma receptors. Allergy. 1999;54(12):1233–51. [PubMed: 10688427]
39. Thye T, Burchard GD, Nilius M, Muller-Myhsok B, Horstmann RD. Genomewide linkage analysis identifies polymorphism in the human interferon-gamma receptor affecting Helicobacter pylori infection. Am J Hum Genet. 2003;72(2):448–53. [PubMed: 12516030]
40. de Albuquerque AC, Rocha LQ, de Morais Batista AH, Teixeira AB, Dos Santos DB, Nogueira NA. Association of polymorphism +874 A/T of interferon-gamma and susceptibility to the development of tuberculosis: meta-analysis. Eur J Clin Microbiol Infect Dis. 2012;31(11):2887–95. [PubMed: 22684265]
41. Zhou J, Chen DQ, Poon VK, Zeng Y, Ng F, Lu L, et al. A regulatory polymorphism in interferon-gamma receptor 1 promoter is associated with the susceptibility to chronic hepatitis B virus infection. Immunogenetics. 2009;61(6):423–30. [PubMed: 19488747]
42. Liu J, Hou Y, Sun L, Li C, Li L, Zhao Y, et al. A pilot study of tofacitinib for refractory Behcet's syndrome. Ann Rheum Dis. 2020.

43. Dudding T, Haworth S, Lind PA, Sathirapongsasuti JF, and Me Research T, Tung JY, et al. Genome wide analysis for mouth ulcers identifies associations at immune regulatory loci. Nat Commun. 2019;10(1):1052. [PubMed: 30837455]

44. Ahn VE, Chu ML, Choi HJ, Tran D, Abo A, Weis WI. Structural basis of Wnt signaling inhibition by Dickkopf binding to LRP5/6. Dev Cell. 2011;21(5):862–73. [PubMed: 22000856]

45. Maruotti N, Corrado A, Neve A, Cantatore FP. Systemic effects of Wnt signaling. J Cell Physiol. 2013;228(7):1428–32. [PubMed: 23359342]

46. Staal FJ, Luis TC, Tiemessen MM. WNT signalling in the immune system: WNT is spreading its wings. Nat Rev Immunol. 2008;8(8):581–93. [PubMed: 18617885]

47. Shi J, Chi S, Xue J, Yang J, Li F, Liu X. Emerging Role and Therapeutic Implication of Wnt Signaling Pathways in Autoimmune Diseases. J Immunol Res. 2016;2016:9392132. [PubMed: 27110577]

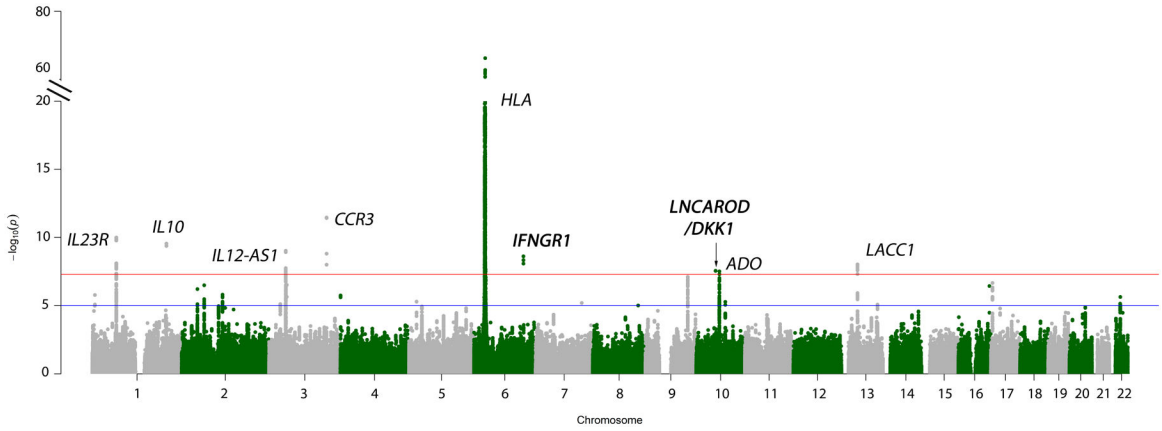

Figure 1. Manhattan plot showing the results of a meta-analysis of Behçet's disease cases and controls included in this study.

The -$\log_{10}$ p value for each genetic variant analyzed is plotted against its physical chromosomal position. The red and blue lines represent the genome-wide level of significance (p value < 5×10^{-8}) and the suggestive level of significance (p value < 5×10^{-5}), respectively.

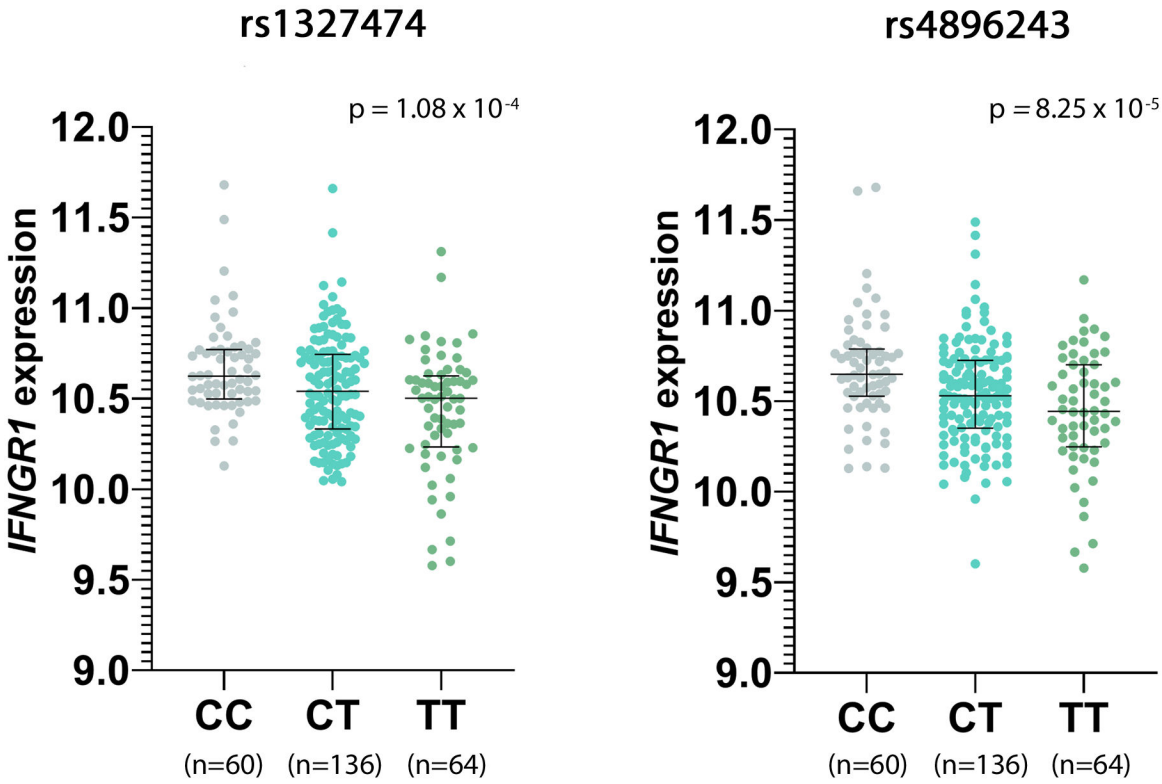

Figure 2. Expression quantitative trait loci (eQTL) associations between two *IFNGR1*-variants (rs1327474 and rs4896243) and *IFNGR1* transcript in 2-hour lipopolysaccharide (LPS) stimulated monocytes.

The risk alleles (C) for both SNPs correlate with significantly higher expression levels of *IFNGR1* (one-way ANOVA p value= 1.08×10^{-4} and 8.25×10^{-5}, respectively). Differences between genotypes were assessed using Tukey's multiple comparisons test; rs1327474 (CC vs. CT, $p = 4.40 \times 10^{-2}$; CC vs. TT, $p = 5.98 \times 10^{-5}$; CT vs. TT = 2.10×10^{-2}), rs4896243 (CC vs. CT, $p = 6.15 \times 10^{-3}$; CC vs. TT, $p = 5.74 \times 10^{-5}$; CT vs. TT = 1.02×10^{-1}).

Table 1.

Results of the meta-analysis for the lead SNP of each genetic region associated at GWAS-level of significance (p value= 5×10^{-8}) in our study. The two new loci are highlighted in bold font.

Locus	Chr	Position (HG19)	SNP	Location	Minor Allele	P value	OR
IL10	1	206945311	rs3024490	Intronic	A	2.81E-10	1.26
Il23R	1	67744601	rs6660226	Downstream	A	1.01E-10	0.79
IL12A-AS1	3	159637678	rs76830965	Intronic	A	3.43E-12	1.66
CCR3	3	46208310	rs2087726	Intronic	G	9.33E-10	0.79
IFNGR1	**6**	**137514790**	**rs4896243**	**Downstream**	**C**	**2.42E-09**	**1.25**
LNCAROD-DKK1	**10**	**54154620**	**rs1660760**	**Intergenic**	**T**	**2.75E-08**	**0.78**
ADO	10	64561506	rs12220700	Upstream	G	3.07E-08	0.80
LACC1	13	44457925	rs2121034	Downstream	T	9.44E-09	0.80

Table 2.

Functional annotation of the 8 *non-HLA* loci associated at GWAS-level of significance (p value= 5×10^{-8}) in our study.

The variant showing the highest RegulomeDB score for each genomic region is displayed.

Locus	SNP	RegulomeDB score	Promoter histone marks	Enhancer histone marks	DNase hyper-sensitivity	Proteins bound	eQTL in blood cells	eQTL in other tissues
IL10	rs1800872	0.609	Yes	Yes		Yes	*IL10*	*IL19, IL24, FAIM3*
Il23R	rs2019262	0.638	Yes				*IL23R*	*MIER1, IL12RB2, C1orf141*
IL12A-AS1	rs76830965	0.775	Yes	Yes	Yes	Yes		*IL12A, TRIM59, BTN3A1, STAT1, GBP1, IFI6, APOL3, IFI44L, HERC6, MX1, GBP2, SCHIP1*
CCR3	rs35678191	0.614		Yes			*CCR5, CCR3, CCR2, CCRL2, CCR9, LZTFL1, CCR1, LRRC2, CXCR6, SACM1L*	*CXCR6, CCR2, CCR1, SLC6A20, PRSS45, PRSS46, CCR5*
IFNGR1	rs4896243	0.805		Yes			*IFNGR1*	*IFNGR1*
LNCAROD-DKK1	rs1660760	0.184						*DKK1*
ADO	rs224106	0.154					*ADO*	*ADO, EGR2*
LACC1	rs2121033	0.922					*CCDC122, LACC1*	*CCDC122, LACC1, ENOX1*