



Published in final edited form as:

Nat Genet. 2010 August ; 42(8): 698–702. doi:10.1038/ng.625.

Genome-wide association study identifies variants in the MHC class I, *IL10*, and *IL23R/IL12RB2* regions associated with Behçet's disease

Elaine F. Remmers¹, Fulya Cosan², Yohei Kirino¹, Michael J. Ombrello¹, Neslihan Abaci³, Colleen Satorius¹, Julie M. Le¹, Barbara Yang⁴, Benjamin D. Korman¹, Aris Cakiris³, Ozgur Aglar³, Zeliha Emrence³, Hulya Azakli³, Duran Ustek³, Ilknur Tugal-Tutkun⁵, Gulsen Akman-Demir⁶, Wei Chen⁷, Christopher I. Amos⁷, Michael B. Dizon⁴, Afet Akdag Kose⁸, Gulsevrim Azizlerli⁸, Burak Erer², Oliver J. Brand⁹, Virginia G. Kaklamani¹⁰, Phaedon Kaklamani¹¹, Eldad Ben-Chetrit¹², Miles Stanford¹³, Farida Fortune¹⁴, Marwen Ghabra¹⁵, William E. R. Ollier¹⁶, Young-Hun Cho¹⁷, Dongsik Bang¹⁸, John O'Shea¹⁹, Graham R. Wallace²⁰, Massimo Gadina⁴, Daniel L. Kastner¹, and Ahmet Gül^{2,3}

¹ Laboratory of Clinical Investigation, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, Maryland, USA

² Istanbul Faculty of Medicine, Division of Rheumatology, Department of Internal Medicine, Istanbul University, Istanbul, Turkey

³ Department of Genetics, Institute for Experimental Medicine, Istanbul University, Istanbul, Turkey

⁴ Translational Immunology Section, Office of Science and Technology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, Maryland, USA

⁵ Istanbul Faculty of Medicine, Department of Ophthalmology, Istanbul University, Istanbul, Turkey

⁶ Istanbul Faculty of Medicine, Department of Neurology, Istanbul University, Istanbul, Turkey

⁷ University of Texas M. D. Anderson Cancer Center, Houston, Texas, USA

⁸ Istanbul Faculty of Medicine, Department of Dermatology, Istanbul University, Istanbul, Turkey

⁹ School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, UK

Users may view, print, copy, download and text and data- mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: http://www.nature.com/authors/editorial_policies/license.html#terms

Correspondence should be directed to E.F.R. (remmerse@mail.nih.gov).

Author Contributions

Study design: E.F.R., F.C., Y.K., M.J.O., B.D.K., D.U., W.C., C.I.A., J.O'S., M.Ga., D.L.K., A.G. **Analysis:** E.F.R., Y.K., M.J.O., B.Y., W.C., C.I.A., M.B.D., G.R.W., M.Ga., D.L.K., A.G. **Sample procurement and data generation:** E.F.R., F.C., Y.K., M.J.O., N.A., C.S., J.M.L., B.Y., B.D.K., A.C., O.A., Z.E., H.A., D.U., I.T.-T., G.A.-D., W.C., C.I.A., M.B.D., A.A.K., G.A., B.E., O.J.B., V.G.K., Ph.K., E.B.-C., M.S., F.F., M.Gh., W.E.R.O., Y.-H.C., D.B., J.O'S., G.R.W., M.Ga., D.L.K., A.G. **Writing:** E.F.R., Y.K., M.J.O., B.Y., C.I.A., J.O'S., M.Ga., D.L.K., A.G.

None of the authors have competing financial interests.

- ¹⁰ Translational Breast Cancer Research Division Hematology/Oncology Northwestern University, Chicago, Illinois, USA
- ¹¹ Department of Rheumatology, Athens Medical Center, Greece
- ¹² Hadassah-Hebrew University Medical Center, Jerusalem, Israel
- ¹³ King's College, London, UK
- ¹⁴ Queen Mary's College, London, UK
- ¹⁵ University Hospital, Damascus, Syria
- ¹⁶ Centre for Integrated Genomic Medical Research, School of Immunity and Infection, University of Manchester, UK
- ¹⁷ Graduate School of Medical Science and Engineering, KAIST, Daejeon, Korea
- ¹⁸ Department of Dermatology, Yonsei University College of Medicine, Seoul, Korea
- ¹⁹ Lymphocyte Cell Biology Section, Molecular Immunology and Inflammation Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, Maryland, USA
- ²⁰ Academic Unit of Ophthalmology, University of Birmingham, UK

Behçet's disease (BD) is a genetically complex disease of unknown etiology, characterized by recurrent inflammatory attacks affecting orogenital mucosa, eyes, and skin. We performed a GWAS with 311,459 SNPs in 1215 BD patients and 1278 healthy controls from Turkey. We confirmed the known HLA-B51 association and identified a second, independent association within the MHC Class I region. We also found one SNP with genome-wide evidence for association within the gene encoding interleukin-10 (*IL10*). A meta-analysis including a total of 2430 cases and 2660 controls established associations with the *IL10* variant (rs1518111, $P=3.54\times 10^{-18}$, odds ratio 1.45 with 95% confidence interval 1.34-1.58) and with a variant located between the interleukin-23 receptor (*IL23R*) and interleukin 12 receptor $\beta 2$ (*IL12RB2*) genes (rs924080, $P=6.69\times 10^{-9}$, odds ratio 1.28 with 95% confidence interval 1.18-1.39). The disease-associated *IL10* variant (rs1518111 A allele) was associated with diminished mRNA expression and low protein production, suggesting novel therapeutic targets for BD.

BD is prevalent in Middle Eastern countries with the highest rate (4 in 1000 individuals) found in Turkey. A genetic contribution to BD is supported by the high sibling recurrence risk ratio (λ_s), estimated from 11.4 to 52.5 in the Turkish population¹. HLA-B51 is the most strongly associated known genetic factor², however it accounts for less than 20% of the genetic risk, even in familial cases³, indicating that genetic factors remain to be discovered. Candidate gene studies and several small GWASs^{4,5} have examined BD genetics, but the studies have been generally underpowered, making interpretation and replication of the results problematic.

We therefore performed a GWAS of 311,459 autosomal SNPs in 1215 BD cases and 1278 healthy controls from Turkey (Fig. 1, Supplementary Fig. 1, and Supplementary Table 1). Only SNP genotype data that met strict quality control standards (see Online Methods) were

included. A principal components method (Online Methods) was used to evaluate population stratification in the cases and the controls. After correction for 6 PCs, λ_{GC} , a measure of genomic inflation, was reduced from 1.06 to 1.05 (Supplementary Fig. 2a, b, c). Correcting for 6 PCs in the absence of the strongly associated MHC region SNPs reduced λ_{GC} from 1.05 to 1.04 (Supplementary Fig. 2d). Given the minimal degree of population stratification, uncorrected data are presented. $P < 5.0 \times 10^{-8}$ was considered genome-wide significance.

The most significantly associated SNPs (P value $< 10^{-44}$) were located on chromosome 6 in the MHC region. To evaluate the contribution of the HLA-B51 type to BD, we determined the *HLA-B* types of 1190 of the cases and 1257 of the controls. Occurrence of the HLA-B51 type (one or two copies) was found in 59.1% of cases and only 29.3% of controls (odds ratio = 3.49 [95% CI = 2.95 to 4.12], $P = 5.47 \times 10^{-50}$). Within the *HLA-B* region the most significantly associated SNPs were located from telomeric to the *HLA-B* coding region to centromeric to *MICA* (encoding MHC Class I chain related sequence A), which has been suggested to be the source of the BD-HLA-B51 association⁶. We found the HLA-B51 type was more strongly associated with disease than was any genotyped SNP (HLA-B51 allele frequency = 0.352 in cases and 0.159 in controls, $P = 1.44 \times 10^{-54}$, Fig. 2). Strong linkage disequilibrium (LD) was observed between HLA-B51 and all the SNPs located from *HLA-B* to more than 62 kb centromeric to the *MICA* gene, despite spanning several blocks of LD (Supplementary Fig. 3). This LD pattern is observed because the HLA-B51 variant is found almost exclusively on a single extended haplotype. This extended haplotype occurred at 0.321 frequency in cases and 0.144 frequency in controls. Interestingly, the identical SNP haplotype, but lacking HLA-B51, occurred in cases and controls equally at 0.04 frequency, suggesting that HLA-B51 is required for the disease association in the *HLA-B* region.

We next performed a conditional logistic regression analysis of the 292 SNPs from the MHC region with allelic Chi-squared $P < 0.0001$, specifying HLA-B51 as a covariate. None of the *HLA-B/MICA* region SNPs remained significantly associated with BD in the conditional analysis (Fig. 2). After accounting for the effect of HLA-B51, three SNPs within the *HLA-A* gene region with disease-associations retained genome-wide significance. The most strongly associated SNP, rs9260997, located 50 kb centromeric to the *HLA-A* gene, had a regressor P value = 5.49×10^{-9} , suggesting a highly significant association independent of the HLA-B51 association. Coincidentally, an association of markers within the *HLA-A* region, independent of HLA-B51, was recently described in Japanese BD patients with attribution of the association to the HLA-A26 allele⁵.

In the association data (Fig. 1) we identified near genome-wide significance for one non-MHC SNP, rs936551 ($P = 5.29 \times 10^{-8}$), located at the telomeric end of the short arm of chromosome 4, within the promoter region of *CPLX1*, which encodes complexin-1, a regulator of exocytosis during vesicle membrane fusion. Additionally, one SNP located within the first intron of the *IL10* gene, rs3024490, was strongly suggestive for association with a P value = 2.22×10^{-7} (Fig. 1). We also identified an additional 66 SNPs from an additional 49 chromosomal regions with suggestive evidence for disease association, $P < 0.0001$ (Supplementary Table 2).

To better evaluate genetic associations within the *CPLX1* and *IL10* gene regions, we genotyped the same case and control samples for additional SNPs from the regions predicted by HapMap CEU data to be in LD with the disease-associated SNP. We also fine-mapped the *IL23R/IL12RB2* region, which was identified in an independent GWAS of Behçet's disease in the Japanese population⁷ and within which we found three SNPs with $p < 0.0001$ (Supplementary Table 2).

In the *CPLX1* region, the additional genotyped SNPs increased the estimated coverage (at r^2 greater than 0.8) of HapMap Phase II SNPs with minor allele frequency greater than 0.05 from 57% to 93%. The most significantly associated SNPs were located in the promoter region of the gene, although none were more significantly associated than rs936551 (Supplementary Fig. 4).

In the *IL10* gene region, we genotyped 27 additional SNPs resulting in coverage of 100% of the HapMap Phase II SNPs with greater than 5% minor allele frequency in the CEU samples. Five SNPs were found strongly associated with BD, with one SNP, rs1518111, with genome-wide significance ($P = 1.88 \times 10^{-8}$, Fig. 3a). The disease-associated SNPs were located in the promoter region and the first, second, and third introns of the gene (Supplementary Fig. 5) and all were in strong LD with one another.

In the *IL23R/IL12RB2* region we genotyped 11 additional SNPs increasing coverage from 58% to 88% of the HapMap Phase II SNPs with greater than 5% minor allele frequency in the CEU samples. No SNPs were found with stronger association than rs924080 from the GWAS analysis (Fig. 3b). We identified a recombination hotspot located in the intergenic region between *IL23R* and *IL12RB2* (Fig. 3b), which is also seen in the HapMap Caucasian and Asian populations (data not shown). The BD-associated variants were located on the *IL23R* side of the hotspot, suggesting the disease association is more likely to influence *IL23R* than *IL12RB2*. Variants within the *IL23R* gene have been associated with ankylosing spondylitis⁸, psoriasis⁹, and inflammatory bowel disease¹⁰, diseases with some phenotypic overlap with each other and with BD. Neither of two *IL23R* coding variants associated with these seronegative diseases was associated with BD (Fig. 3b).

We genotyped the BD-associated *IL10*, *IL23R/IL12RB2*, and *CPLX1* SNPs in five additional collections of BD cases and controls from Turkey, the Middle East, Europe, and Asia (see Table 1 and Online Methods). A surrogate marker (rs11248047) was used in the place of the *CPLX1* disease-associated SNP (rs936551), which failed quality control in the replication assay (both markers shown in Supplementary Fig. 4). The results are shown in Table 1. A number of the UK Caucasian and Middle Eastern Arab samples were previously tested for association of two *IL10* promoter region SNPs with BD. Although an association with disease was reported in the UK collection, the association had not replicated in the Middle Eastern Arab collection¹¹. The BD-associated *IL10* SNP identified in the Turkish population, rs1518111, reached statistical significance after correction for three tests ($P < 0.017$) in the Middle Eastern Arab and Greek collections and was of nominal significance ($P < 0.05$) in the UK Caucasian collection (Table 1). For the most part, the *IL23R/IL12RB2* and *CPLX1* SNPs showed the same trend as found in the Turkish samples; however the associations did not reach statistical significance in these smaller collections. Association of

these markers was not observed in the small Turkish replication collection. Although this lack of association could be explained by small sample size, an alternative explanation could be that the markers are associated with severe disease, as we have noted that disease manifestations in the replication collection (drawn from a Dermatology clinic) were milder than in the discovery collection used for the GWAS, which was obtained from a Rheumatology clinic.

While analyzing these data we learned that another group had also performed a GWAS for Behçet's disease⁷. We exchanged data for cross validation purposes and found that the *IL10* and the *IL23R/IL12RB2* SNP associations were also strongly supported by the data obtained from the Japanese population, whereas the *CPLX1* association was not observed. A meta-analysis of all the association data (including a total of 2430 BD cases and 2660 controls) provided strong evidence for associations of the *IL10* and *IL23R/IL12RB2* loci with BD (Table 1). Association of the *CPLX1* variant failed to replicate in any of the additional collections and failed to reach genome-wide significant association in a meta-analysis of the data. Analysis of even larger numbers of replication samples will be required to establish whether the *CPLX1* locus contributes to BD susceptibility.

Several SNP haplotypes of the *IL10* gene promoter have been reported to be associated with regulation of the gene's expression, but the reported allelic effects have been inconsistent and difficult to reconcile¹²⁻¹⁴. We therefore tested for a difference in expression of the gene from the disease-associated haplotype and other haplotypes by measuring allelic imbalance of the rs1518111 variant in pre-mRNA from monocytes isolated from 8 healthy individuals heterozygous for the disease-associated allele. In 8 donors, the pre-mRNA transcript with the rs1518111 A allele was found at reduced levels compared with the G allele. Expression from the A bearing chromosome was 35% of expression from the G bearing chromosome (Fig. 4a).

To assess whether this expression difference was relevant in terms of cytokine production, we activated mononuclear cells from healthy Turkish donors with lipopolysaccharide (LPS) and found significantly lower amounts of IL-10 protein in supernatants from donors homozygous for the rs1518111 A allele compared with individuals with one or two G alleles (Fig. 4b). We also cultured monocytes from ethnically diverse healthy donors from the United States and stimulated them with two Toll-like receptor and nucleotide-binding oligomerization domain containing receptor ligands, the lipoprotein, Pam₃Cys and muramyl dipeptide. Individuals homozygous for the BD-associated rs1518111 A allele produced significantly less IL-10 than individuals with one or two G alleles (Fig. 4c), whereas no statistically significant variation was found in TNF α production (data not shown).

Taken together, these data suggest that a genetic predisposition for low IL-10 expression is a risk factor for BD. There are extensive data in mouse models linking reduction in IL-10 with inflammation¹⁵. Variants in the *IL10* gene region have been shown to be associated with ulcerative colitis (UC)¹⁶, type I diabetes¹⁷, systemic lupus erythematosus¹⁸, and severe juvenile rheumatoid arthritis (JRA)¹², and IL-10 receptor mutations were recently shown to cause early onset enterocolitis¹⁹. Interestingly, the SNP found in the UC, type I diabetes, and lupus studies, rs3024505, was not associated with BD (Fig. 3a) and the BD-associated *IL10*

variants were not associated with these three diseases, suggesting a different IL-10-related disease mechanism in BD compared with these other diseases. On the other hand, the *IL10* association with severe JRA is the same as for BD.

Variants from several candidate genes have been examined for association with BD. Although no variants within these genes were found with genome-wide significant association in the Turkish GWAS, 6 of the 12 genes examined contained at least one SNP with nominal significance ($P < 0.05$, Supplementary Table 3). A much larger sample collection will be required to determine whether any of these genes harbor true BD susceptibility alleles.

In summary, this GWAS provides new insights into the genetic factors that contribute to BD. It not only supports the association of HLA-B51 as the primary MHC association with BD, but also reveals another independent MHC Class I association telomeric to the *HLA-B* gene. Most importantly, this study demonstrates that common variations in the *IL10* gene and in the vicinity of the *IL23R/IL12RB2* genes predispose to BD. Functional analyses indicate that the disease-associated *IL10* variants cause decreased expression of this anti-inflammatory cytokine, and thereby, possibly in concert with commensal microorganisms²⁰, result in an inflammation-prone state, thus suggesting a mechanistic hypothesis and possible therapeutic targets.

Data accession

The genotype data for the 311,459 SNPs in 1215 Behçet's disease cases and 1278 healthy controls from Turkey have been deposited in the National Institutes of Health database of genes and phenotypes, dbGaP (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gap>), accession number: phs000272.v1.p1

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the Intramural Research Program of the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health, by the Istanbul University Research Fund, and by the UK Behçet's Syndrome Society. We thank all of our patients for their enthusiastic support of our studies aiming to understand and find better treatments for Behçet's disease, and Ms. Omur Aksakalli and Ms. Hatice Ustek for their help in blood collection.

Online Methods

Patients and healthy controls

GWAS samples were collected at the Behçet's Disease Clinic of Istanbul Faculty of Medicine, Division of Rheumatology, one of the largest centers for BD in Turkey, as consecutive patients fulfilling the International Study Group Criteria²¹. Demographic and clinical characteristics of the patients are given in Supplementary Table 1. Healthy controls were selected among blood bank donors or healthy volunteers to match by geographic

location the place of birth of the patients (Supplementary Fig. 1). All healthy controls were questioned for the absence of recurrent oral aphthous ulcers or other BD-related manifestations, or a family history for BD. All study participants provided written informed consent, and the study was approved by the Ethics Committee of the Istanbul Faculty of Medicine.

The replication study samples were from 1) a non-overlapping set of Turkish cases and controls obtained from the Department of Dermatology Behçet's Disease Clinic of the Istanbul Faculty of Medicine, 2) a collection of BD cases and controls of Middle Eastern Arab origin¹¹, 3) a collection of Greek cases and controls²², 4) a collection of UK Caucasian BD cases and controls¹¹, and 5) a collection of Korean BD cases and controls collected at Yonsei University College of Medicine in Seoul, South Korea. All cases fulfilled the International Study Group Criteria.

DNA preparation

Blood samples were collected from all participants and genomic DNA was isolated using the MagNA Pure Instrument with the MagNA Pure Compact DNA Isolation Kit for 1 ml volume (Roche, Mannheim, Germany).

Whole genome SNP typing, quality control, and association analysis

All DNA samples were typed using the Infinium assay (Illumina). The first 332 (146 cases and 186 controls) samples were typed on Human CNV370-Duo v1.0 chips. The remaining 2329 samples (1175 cases and 1150 controls) were typed on Human CNV370-Quad v3.0 chips. Samples with high call rates using Illumina-provided HapMap clusters (greater than 99% on Duo chips and greater than 99.5% on Quad chips) were clustered and these cluster locations were used to determine the genotypes of all the samples. All 2657 samples (1321 cases and 1336 controls) had > 95% call rate. SNPs used in the analysis were located on the autosomes, had > 95% call rate, > 1% minor allele frequency, and Hardy Weinberg equilibrium p -value > 0.00001 determined separately with Duo and Quad chip data. There were 335,887 autosomal SNPs on the Duo and 334,556 SNPs on the Quad chips. Filtering removed 9436 SNPs from the Duo and 11,886 SNPs from the Quad chip data. The Duo and Quad data were merged omitting SNPs not present on both chips (14,970 non-overlapping SNPs from the Duo and 11,188 from the Quad chips). The merged, autosomal data included 311,482 SNPs. Heterozygosity of X chromosome SNPs was used to predict the sex of the samples. We removed 19 samples with sex mismatch. PLINK²³ was used to identify samples with genetic relatedness indicating identity (or monozygotic twin) or first, second, or third degree relatives. Only one of each pair of identical samples was included; if however the identity could not be confirmed, both samples were removed. Only one of each pair of first to third degree related samples was included, selecting the sample that was the case or the one with the highest call rate. A total of 145 samples were removed for relatedness. The samples used in the genome-wide association analysis included 1215 cases and 1278 controls. The SNP genotypes from these 2493 individuals were refiltered using the same criteria (> 95% call rate, > 1% minor allele frequency, and Hardy-Weinberg equilibrium > 0.00001) resulting in 311,459 SNPs in the final analysis.

The SNP data were analyzed for SNP association using Sequence Variation Suite 7 (SVS7) software (Golden Helix, Bozeman, Montana). A principal components analysis method was used to detect possible stratification present in the cases and the controls. The genomic inflation factor λ_{GC} was 1.06 before correction for the top 6 principal components and 1.05 after correction, and without the MHC region markers was 1.05 without correction and 1.04 with correction for 6 PCs (Supplementary Fig. 2).

Fine map genotyping

Additional SNPs were selected for genotyping in the regions of LD surrounding the disease-associated SNPs identified by the genome-wide association analysis. We used the Tagger algorithm in Haploview²⁴ to select SNPs to augment the coverage of the GWAS SNPs to obtain 100% coverage of the HapMap Phase II SNPs with greater than 5% minor allele frequency in the CEU HapMap population with pairwise $r^2 > 0.8$. Genotyping of the same samples used for the GWAS was performed using the Sequenom iPLEX gold method (Sequenom, San Diego, California). Case and control DNAs were run at the same time and genotypes determined using Typer 4.0 software (Sequenom). Genotype clusters were edited from the combined case and control data without knowledge of the case/control status of individual samples. Of the 30, 13, and 47 SNPs added respectively to the *IL10*, *IL23R*, and *CPLX1* regions, 26, 11, and 30 SNPs passed quality control. The concordance rate for SNPs genotyped on the Illumina chip and Sequenom iPLEX platforms was 99.83%.

Replication sample genotyping

Replication samples were genotyped for rs1518111, rs924080, and rs11248047 with TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, California) using the manufacturer's protocol and recommended quality control measures. Genotype clusters were edited from the combined case and control data without knowledge of the case/control status of individual samples.

HLA-B type analysis

HLA-B types were determined from case and control DNA samples using a reverse sequence-specific oligonucleotide (SSO) method (One Lambda, Canoga Park, California) and a Bio-Plex 200 suspension array system (BioRad, Hercules, California). Samples were scored for 0, 1, or 2 HLA-B*51 alleles.

Preparation of PBMCs and monocytes from peripheral blood

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by centrifugation through Ficoll-Hypaque solution (Biocoll Separation solution cat: L6115, BioChrome AG, Berlin, Germany) and washed twice in phosphate buffered saline (PBS). Cells were resuspended in culture medium at a concentration of 1×10^6 cells per ml. Cells were cultured in complete RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (cat. SH3008803, Thermo Scientific, Waltham, Massachusetts), 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 ug/mL) (cat. 15670-063, Invitrogen) at 37°C in a humidified atmosphere containing 5% CO₂ (Thermo Scientific). Monocytes were obtained

from PBMCs by negative selection (Monocyte Isolation Kit II; Miltenyi Biotec, Gladbach, Germany). The monocytes were washed and cultured at 2.5×10^5 cells per ml in RPMI 1640 containing 5% FBS.

Assay for IL10 allelic imbalance

A genotype assay for rs1518111 (C___8828803_1_, Applied Biosystems, Foster City, California), an intronic SNP within the *IL10* gene, was used in a quantitative expression assay to compare the relative expression of the disease-associated and the other copy of the *IL10* gene in pre-mRNA from heterozygous carriers of the disease-associated haplotype. Monocytes were isolated from heterozygous carriers of the *IL10* disease-associated haplotype. Total RNA was treated with DNase I (Invitrogen) prior to synthesis of cDNA, which was used in the assay for relative expression of the two copies of the gene. The expression data were analyzed in triplicate and corrected for the detection difference of the two alleles using genomic DNA from each donor. The relative expression of the disease-associated allele compared with other alleles was determined using the 2^{-Ct} method:

Relative expression of the A allele compared with the G allele = 2^{-Ct}

$$Ct = (\text{Avg FAM (A) Ct cDNA} - \text{Avg FAM (A) Ct gDNA}) - (\text{Avg VIC (G) Ct cDNA} - \text{Avg VIC (G) Ct gDNA}).$$

The significance of the difference in expression of the A allele relative to the G allele was determined with a Wilcoxon matched pairs signed rank test with $P < 0.05$ considered significant.

Assay for IL-10 in supernatants of stimulated PBMCs or monocytes isolated from peripheral blood

For LPS-stimulated PBMCs, LPS (*E. coli*, Serotype O26:B6, cat. L-3274, Sigma Aldrich, St. Louis, Missouri) was added to the cells ($1 \mu\text{g}/10^6$ cells) and incubated 24 hours. Supernatants were collected, and IL-10 concentration was analyzed by enzyme-linked immunosorbent assay (ELISA) with a commercially available kit according to manufacturer's protocol (Human IL-10 ELISA Kit, cat. KHC0101, Invitrogen). Absorbance was measured at 450 nm in a DTX 800 Multimode Detector (Beckman Coulter, Brea, California). The assay detection limit was 7.8 pg/ml.

For MDP + Pam₃Cys-stimulated monocyte experiments, cells were stimulated for 24 hours with medium, or medium supplemented with MDP (10 $\mu\text{g}/\text{ml}$; Sigma Aldrich) and Pam₃Cys (1 $\mu\text{g}/\text{ml}$; InvivoGen, San Diego, California). Supernatants were collected and concentrations of IL-10 and TNF- α were measured with the Bio-Plex Pro Human Cytokine x-plex assay. Data were collected using the Bio-Plex 200 system according to the manufacturer's protocol (Bio-Rad).

A Mann-Whitney test was used to test for significant differences among genotypes with $P < 0.05$ considered significant. For LPS-stimulated PBMC, 11 homozygotes for the A allele, 13 heterozygotes, and 14 homozygotes for the G allele were used. For the MDP + Pam₃Cys

stimulated monocytes, 11 homozygotes for the A allele, 18 heterozygotes, and 18 homozygotes for the G allele were used.

References

1. Gul A, Inanc M, Ocal L, Aral O, Konice M. Familial aggregation of Behcet's disease in Turkey. *Ann Rheum Dis.* 2000; 59:622–5. [PubMed: 10913059]
2. 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; 61:1287–96. [PubMed: 19790126]
3. Gul A, et al. Evidence for linkage of the HLA-B locus in Behcet's disease, obtained using the transmission disequilibrium test. *Arthritis Rheum.* 2001; 44:239–40. [PubMed: 11212166]
4. Fei Y, et al. Identification of novel genetic susceptibility loci for Behcet's disease using a genome-wide association study. *Arthritis Res Ther.* 2009; 11:R66. [PubMed: 19442274]
5. Meguro A, et al. Genetics of Behcet's disease inside and outside the MHC. *Ann Rheum Dis.* 2010; 69:747–754. [PubMed: 19684014]
6. Mizuki N, et al. Triplet repeat polymorphism in the transmembrane region of the MICA gene: a strong association of six GCT repetitions with Behcet disease. *Proc Natl Acad Sci U S A.* 1997; 94:1298–303. [PubMed: 9037047]
7. Mizuki N. Pre-publication reference. *Nat Genet.* In press.
8. Burton PR, et al. Association scan of 14,500 nonsynonymous SNPs in four diseases identifies autoimmunity variants. *Nat Genet.* 2007; 39:1329–37. [PubMed: 17952073]
9. Cargill M, et al. A large-scale genetic association study confirms IL12B and leads to the identification of IL23R as psoriasis-risk genes. *Am J Hum Genet.* 2007; 80:273–90. [PubMed: 17236132]
10. Duerr RH, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science.* 2006; 314:1461–3. [PubMed: 17068223]
11. Wallace GR, et al. IL-10 genotype analysis in patients with Behcet's disease. *Hum Immunol.* 2007; 68:122–7. [PubMed: 17321902]
12. Crawley E, et al. Polymorphic haplotypes of the interleukin-10 5' flanking region determine variable interleukin-10 transcription and are associated with particular phenotypes of juvenile rheumatoid arthritis. *Arthritis Rheum.* 1999; 42:1101–8. [PubMed: 10366102]
13. Temple SE, et al. Alleles carried at positions -819 and -592 of the IL10 promoter affect transcription following stimulation of peripheral blood cells with *Streptococcus pneumoniae*. *Immunogenetics.* 2003; 55:629–32. [PubMed: 14605776]
14. Turner DM, et al. An investigation of polymorphism in the interleukin-10 gene promoter. *Eur J Immunogenet.* 1997; 24:1–8. [PubMed: 9043871]
15. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol.* 2001; 19:683–765. [PubMed: 11244051]
16. Franke A, et al. Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. *Nat Genet.* 2008; 40:1319–23. [PubMed: 18836448]
17. Barrett JC, et al. Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes. *Nat Genet.* 2009
18. Gateva V, et al. A large-scale replication study identifies TNIP1, PRDM1, JAZF1, UHRF1BP1 and IL10 as risk loci for systemic lupus erythematosus. *Nat Genet.* 2009; 41:1228–33. [PubMed: 19838195]
19. Glocker EO, et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med.* 2009; 361:2033–45. [PubMed: 19890111]
20. Rakoff-Nahoum S, Hao L, Medzhitov R. Role of toll-like receptors in spontaneous commensal-dependent colitis. *Immunity.* 2006; 25:319–29. [PubMed: 16879997]
21. International_Study_Group_for_Behcet's_Disease. Criteria for diagnosis of Behcet's disease. International Study Group for Behcet's Disease. *Lancet.* 1990; 335:1078–80. [PubMed: 1970380]

22. Kaklamani VG, Sadim M, Koumantaki Y, Kaklamanis P, Pasche B. Role of polymorphisms in Adamantiades-Behcet's disease. *J Rheumatol.* 2008; 35:2376–8. [PubMed: 18925687]
23. Purcell S, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet.* 2007; 81:559–75. [PubMed: 17701901]
24. Barrett JC. Haploview: Visualization and analysis of SNP genotype data. *CSH Protoc.* 2009; 2009 pdb ip71.

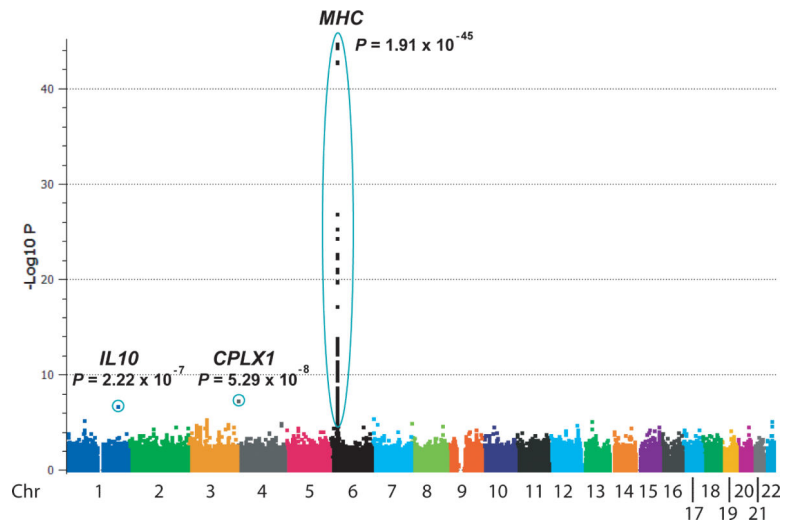


Figure 1. Behçet's disease genome-wide association results. The $-\log_{10} P$ values (allelic Chi-squared test) for association of 311,459 autosomal SNPs in 1215 BD cases and 1278 controls from Turkey are shown segregated by chromosome and sorted by genomic position.

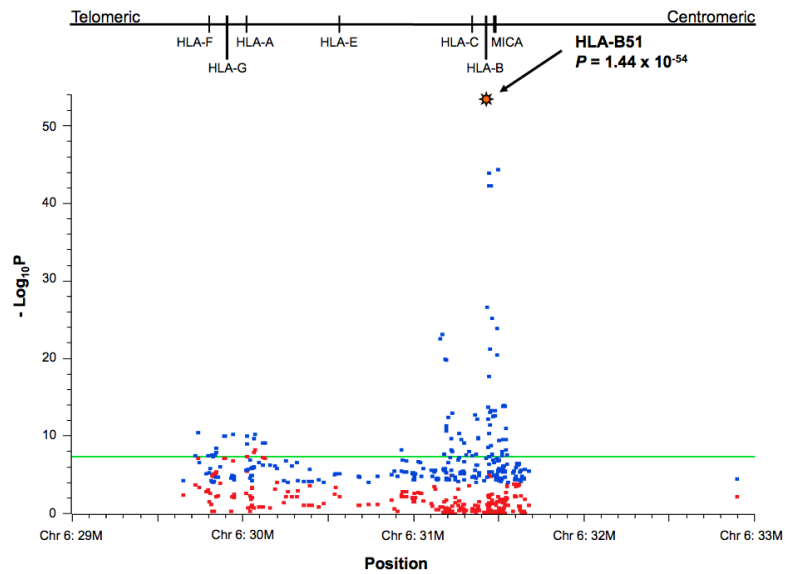
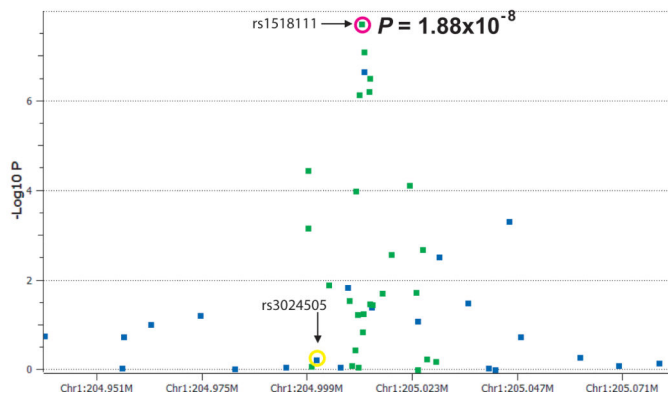
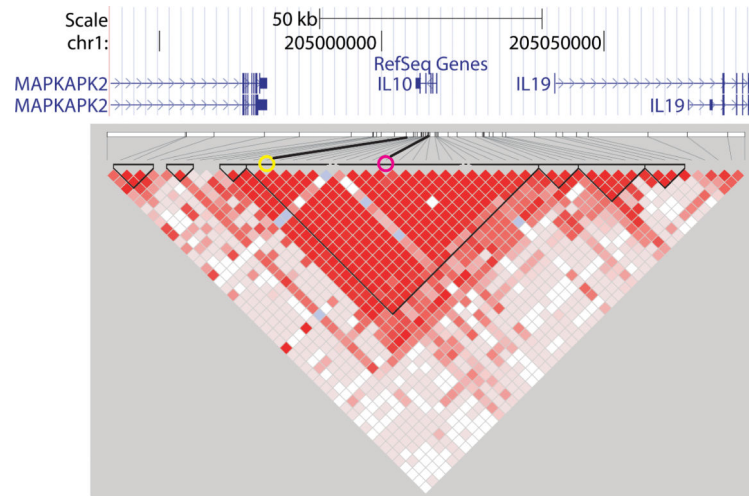


Figure 2.

Analysis of associations within the MHC. The 292 SNPs from the MHC region with allelic Chi-squared $P < 0.0001$ are shown before (blue symbols) and after (red symbols) conditioning for HLA-B51. Blue symbols represent $-\log_{10} P$ (allelic Chi-squared test). Red symbols represent $-\log_{10}$ regressor P -values of the same markers from a logistic regression analysis with HLA-B51 specified as a covariate. The green horizontal line at $-\log_{10} P = 7.301$ corresponds to the genome-wide significance threshold of 5×10^{-8} . The locations of *HLA* genes are shown above the association graph.



Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

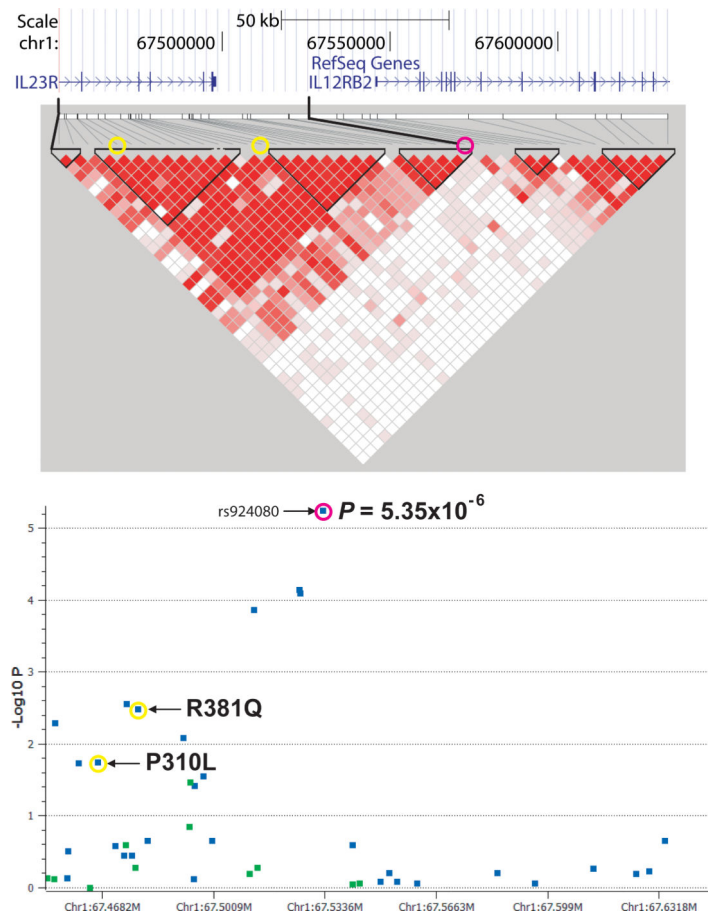
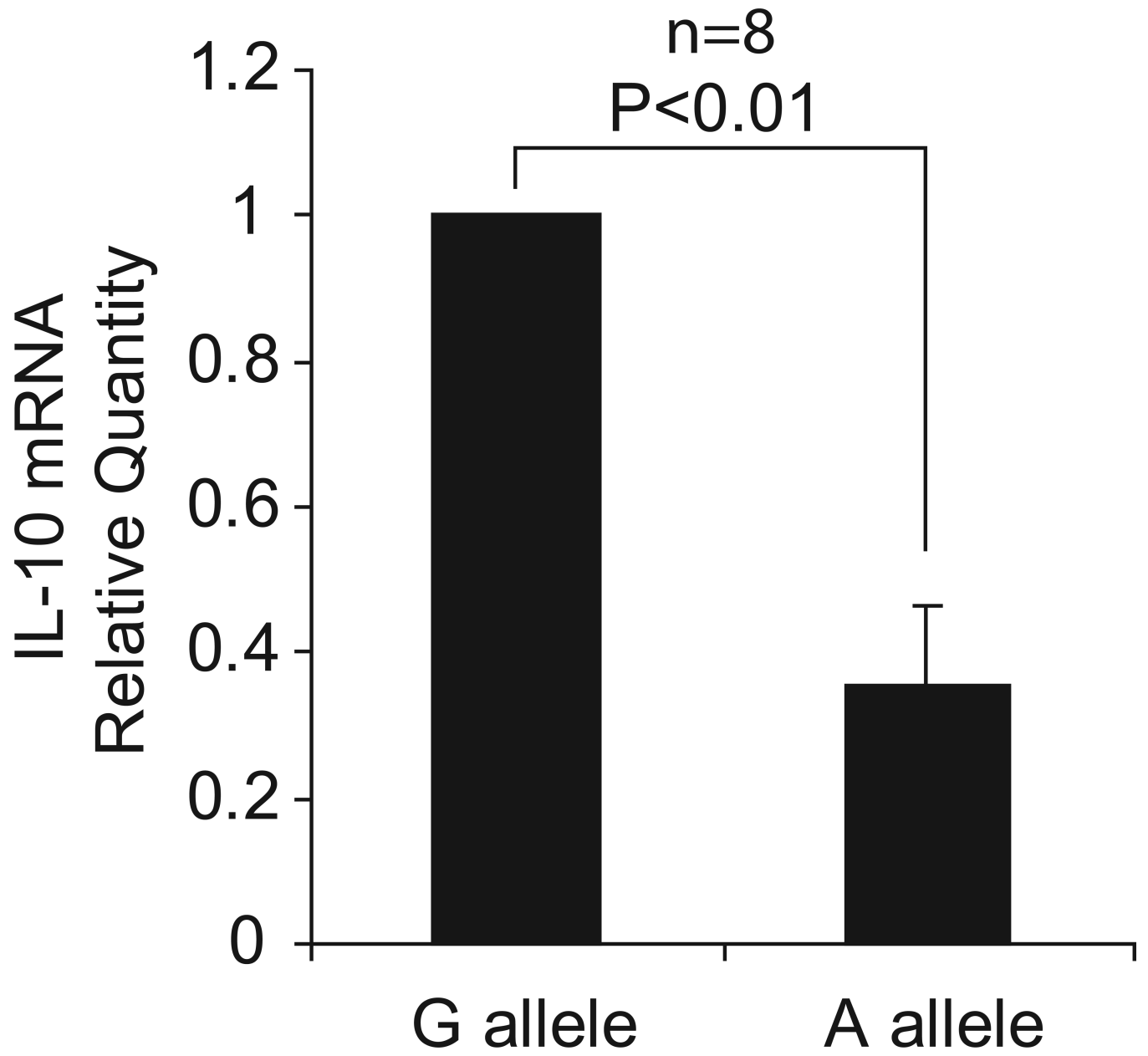
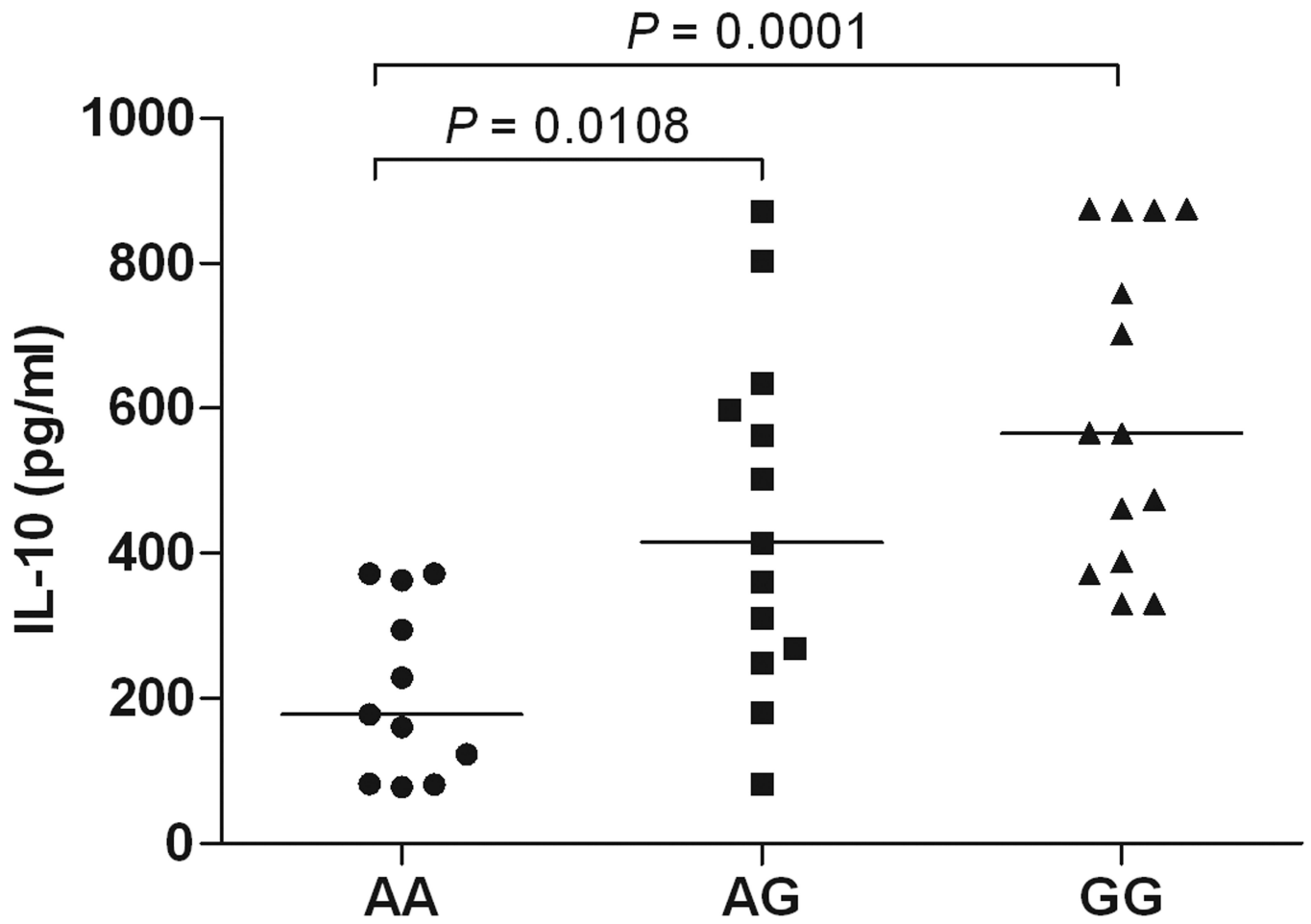


Figure 3.

Fine-mapping of the *IL10* and *IL23R/IL12RB2* regions. Regional association plot and linkage disequilibrium structure of the disease-associated regions surrounding (a) *IL10* and (b) *IL23R/IL12RB2* showing SNPs genotyped in the genome-wide analysis (blue) and fine-mapping analysis (green). The LD structure of the same regions are shown with red filled squares linking pairs of markers indicating the intensity of LD by intensity of fill, $D' = 1$ (intense red) to $D' = 0$ (no fill). In (a) the pink circles represent rs1518111 and the yellow circles represent rs3024505, which is associated with IBD and SLE. In (b) the pink circles represent rs924080 and the yellow circles represent the *IL23R* coding variants associated with other seronegative diseases.





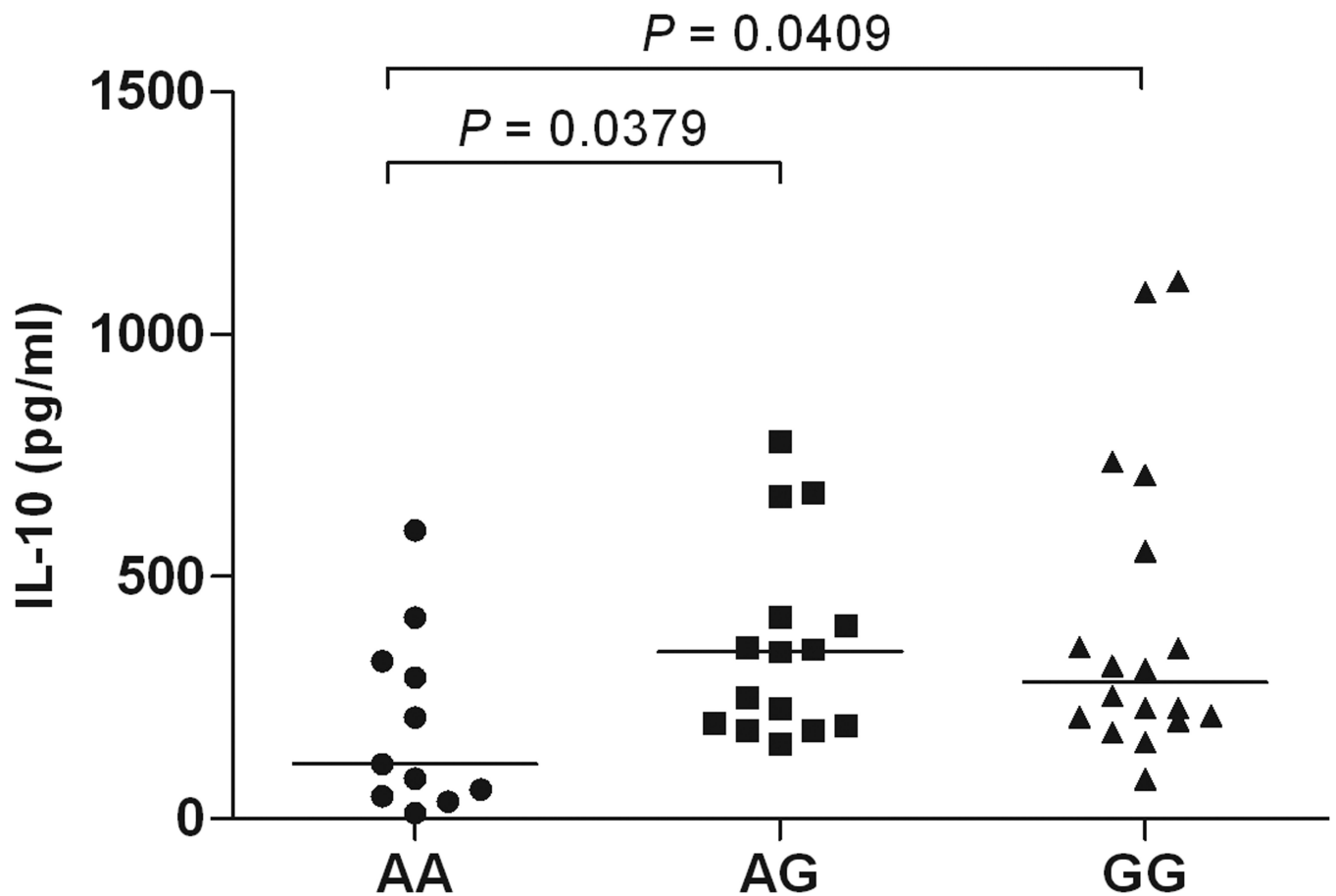


Figure 4.

Expression analysis of *IL10*. (a) Allelic imbalance detected by real time PCR of the *IL10* SNP, rs1518111 (A is the BD-associated allele), in pre-mRNA from purified monocytes from healthy, rs1518111 heterozygous blood donors (n=8, p<0.01). The error bar represents the standard deviation. (b) IL-10 production by peripheral blood mononuclear cells isolated from healthy Turkish blood donors measured 24 hr after stimulation with LPS, relative to rs1518111 genotype. (c) IL-10 production by CD14+ monocytes isolated from ethnically diverse, healthy American blood donors measured 24 hr after stimulation with MDP + PAM₃Cys, relative to rs1518111 genotype. In (b) and (c) the horizontal lines indicate the medians.

Table 1

Association of three SNPs identified in the discovery GWAS in independent collections of BD cases and controls from diverse ethnic backgrounds.

Sample Collection	# cases	# controls	Allele freq cases	Allele freq ctrls	Chi-squared	ChiSq P-value	Odds ratio	(95% CI)
<i>rs1518111 (IL10) A/G</i>								
Discovery - Turkish	1161	1221	0.38	0.30	31.62	1.88E-08 *	1.41	(1.25 - 1.59)
Replication - Turkish	110	224	0.30	0.31	(0.02)	0.689	0.97	(0.69 - 1.38)
Replication - Middle Eastern Arab	188	163	0.35	0.26	7.15	0.007	1.56	(1.12 - 2.16)
Replication - Greek	107	84	0.40	0.27	7.21	0.007	1.41	(1.26 - 1.59)
Replication - UK Caucasian	120	119	0.31	0.22	4.49	0.034	1.56	(1.03 - 2.34)
Replication - Korean	77	52	0.71	0.66	0.57	0.450	1.23	(0.78 - 2.10)
Replication - Japanese	611	737	0.76	0.67	29.40	5.89E-08	1.60	(1.35 - 1.90)
CMH-Meta-analysis	2374	2600			75.56	3.54E-18 *	1.45**	(1.34 - 1.58)
<i>rs924080 (IL23R/IL12RB2) A/G</i>								
Discovery - Turkish	1213	1275	0.67	0.61	20.71	5.35E-06	1.31	(1.17 - 1.47)
Replication - Turkish	110	220	0.64	0.65	(0.05)	0.818	0.96	(0.69 - 1.35)
Replication - Middle Eastern Arab	165	146	0.65	0.59	2.60	0.107	1.31	(0.94 - 1.81)
Replication - Greek	102	82	0.58	0.58	0.00	0.956	1.01	(0.67 - 1.53)
Replication - UK Caucasian	119	118	0.55	0.53	0.30	0.585	1.11	(0.77 - 1.59)
Replication - Korean	77	54	0.79	0.74	0.72	0.397	1.28	(0.72 - 2.28)
Replication - Japanese	597	720	0.76	0.70	13.54	2.34E-04	1.39	(1.17 - 1.65)
CMH-Meta-analysis	2383	2615			33.62	6.69E-09 *	1.28**	(1.18 - 1.39)
<i>rs11248047 (CPLX1) G/A</i>								
Discovery - Turkish	1176	1190	0.58	0.50	27.93	1.26E-07	1.36	(1.21 - 1.53)
Replication - Turkish	111	225	0.54	0.54	0.00	0.967	0.99	(0.72 - 1.37)
Replication - Middle Eastern Arab	189	163	0.60	0.56	0.68	0.409	1.13	(0.84 - 1.53)
Replication - Greek	104	84	0.55	0.51	0.49	0.485	1.16	(0.77 - 1.74)
Replication - UK Caucasian	116	115	0.55	0.55	0.01	0.933	1.02	(0.70 - 1.47)
Replication - Korean	75	51	0.54	0.50	0.39	0.533	1.17	(0.71 - 1.94)
Replication - Japanese	610	726	0.46	0.47	(0.58)	0.445	0.94	(0.81 - 1.10)
CMH-Meta-analysis	2381	2554			13.82	0.0002	1.16**	(1.07 - 1.27)

* Genome-wide significance ($P < 5.0E-08$).

*** Breslow-Day Test for Homogeneity of the Odds Ratios: P =0.54, 0.71, and 0.027 for rs1518111, rs924080, and rs11248047, respectively.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript