

Variants of the *IFI16* Gene Affecting the Levels of Expression of mRNA Are Associated with Susceptibility to Behçet Disease

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ABSTRACT. Objective. Behçet disease (BD) is a multifactorial disease in which infectious agents have been proposed as triggers in genetically predisposed individuals. The aim of our study was to investigate the role of innate immunity receptors, specifically the nucleic acid sensors, in susceptibility to BD.

Methods. Seventy-four tag single nucleotide polymorphisms (tSNP) selected in 9 candidate genes (*DDX58*, *IFIH1*, *TLR3*, *TLR7*, *TLR8*, *AIM2*, *IFI16*, *ZBP1*, and *TLR9*) were genotyped in 371 patients and 854 controls. Assays of mRNA expression and allele-specific transcript quantification (ASTQ) were performed in 110 and 50 controls, respectively.

Results. Patients and controls were genotyped and 2 tSNP (rs6940 in *IFI16* and rs855873 in *AIM2*) were associated with BD. To confirm this association, these tSNP were genotyped in 850 additional controls, and the total cohort was randomly divided into 2 cohorts. The association of these 2 tSNP with the disease remained in both cohorts. One haplotype (rs6940T-rs855873G) was identified as a risk factor (OR 1.41, 95% CI 1.06–1.86, $p = 0.015$), and another (rs6940A-rs855873A) as a protective factor (OR 0.65, 95% CI 0.47–0.90, $p = 0.009$). Samples with the risk haplotype had lower *IFI16* expression levels than samples with the protective (0.99 ± 0.29 vs 1.23 ± 0.50 , $p = 0.022$). Consistently, in the ASTQ assays performed with the nonsynonymous rs6940 SNP, the risk allele had lower *IFI16* expression levels than the protective ($p = 0.027$).

Conclusion. Our findings suggest association of *IFI16*, a cytosolic sensor of dsDNA and mediator of the AIM2 inflammasome-dependent pathway, in susceptibility to BD. Differences genetically determined in the levels of this molecule could be the cause of this association. (First Release Feb 1 2015; *J Rheumatol* 2015;42:695–701; doi:10.3899/jrheum.140949)

Key Indexing Terms:

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Behçet disease (BD) is a systemic vasculitis mainly characterized by recurrent oral and genital ulceration, although other clinical manifestations such as skin lesions, ocular, gastrointestinal, and neurological disorders are relatively common¹. The prevalence depends on the geographic location of the study population. The highest corresponds to Turkey (80 to 420 cases per 100,000 people), followed by Japan (70–85/100,000). Among Western countries, the Mediterranean basin has the highest prevalence (e.g., Spain has 0.7/100,000)^{1,2}. Although the etiology of BD remains unclear, it is known that it is an immune-mediated disease in which imbalances in the innate or adaptive immune response, triggered by infectious agents or environmental factors in genetically predisposed individuals, may be some of the underlying mechanisms of this multifactorial disease³. Regarding the environmental factors, infectious agents of the oral microbial flora (oral aphthae are the first manifestation in most patients) have been proposed as triggers of the disease⁴. Regarding the genetic factors, *HLA-B51* has been the most consistently associated with the disease. Nevertheless, the contribution of the *HLA* region represents about 20% of the genetic component^{5,6} and other non-*HLA* genes such as interleukin 23R (*IL-23R*) and *IL-10*, related to innate immunity, have been associated more recently with this pathology^{7,8}.

The innate immune system, which is the first barrier against pathogens, uses sensor molecules known as pattern recognition receptors (PRR) to recognize highly conserved structures expressed by microorganisms (pathogen-associated molecular patterns; PAMP) or by damaged cells (damage-associated molecular patterns; DAMP). Some of the PRR recognize components such as nucleic acids and therefore their activation creates, at least potentially, a risk of developing autoimmune disease⁹. Among the PRR involved in the recognition of nucleic acid molecules transported to the endosomal compartment by endocytosis or autophagy, Toll-like receptors (TLR) 3, TLR7, TLR8, and TLR9 are the best known. TLR3 recognizes double-stranded RNA (dsRNA), TLR7, and TLR8 single-stranded RNA (ssRNA) and TLR9 unmethylated dinucleotide CpG motifs of viral and bacterial DNA. After binding of the TLR to their ligands, the transduction signal produces activation of different molecules such as interferon regulatory factors (IRF) 3, IRF7, activating protein 1 and nuclear factor- κ B (NF- κ B), and at the end, transcription of inflammatory cytokines and type I interferon (IFN)¹⁰. Other sensors such as the RIG-I-like receptor (RLR) family, which include DDX58 and IFIH1, detect dsRNA in the cytosol. Similar to TLR, RLR induce activation of IRF3, IRF7, and NF- κ B and trigger the transcription of type I IFN¹¹. Besides the RNA sensors, a large number of sensors of intracellular DNA are

known. ZBP1 acts as a cytosolic dsDNA sensor that initiates IFN responses through activation of the NF- κ B and IRF3 pathways¹¹. IFI16 and AIM2 are cytosolic sensors for dsDNA members of AIM2-like receptors (ALR)^{12,13}; nevertheless, IFI16 and AIM2 proteins have different effects¹⁴. The IFI16 protein stimulates the expression of type I IFN (IFN- β) by activation of IRF3 and NF- κ B¹², and the AIM2 protein activates caspase-1 and leads to inflammation by the cleavage of inflammatory cytokine proforms, such as IL-1 β , IL-18, and IL-33^{15,16,17}. A schematic overview of the different aforementioned PRR, their ligands, and the signal transduction pathways is shown in Figure 1.

It has been proposed that certain infectious agents, especially those in the oral microbial flora, act as triggers of BD. Therefore, it is possible that variations in PRR influence the outcome of BD. The aim of our present study was to analyze the possible relationship between nucleic acid sensors in susceptibility to BD.

MATERIALS AND METHODS

Patients and controls. A total of 371 BD-unrelated patients (45% men) who fulfilled the 1990 International Study Group classification criteria for BD¹⁸ and 2 cohorts of 854 and 850 ethnically matched healthy unrelated bone marrow donors (50% men) were included in the study. All the subjects were Spanish whites recruited from different Spanish hospitals. All local ethics committees of the corresponding hospitals approved the study and all participants in the study gave written informed consent. Mean age at diagnosis was 38.7 \pm 13.8 years and 100% of the patients had oral ulcers, 64% genital ulcers, 59% uveitis, and 48% arthritis. Twenty percent had vascular, 22% neurological, and 19% gastrointestinal involvement.

Peripheral blood was obtained from the healthy controls, whereas peripheral blood or saliva was the starting material obtained from patients. Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's recommendations and stored at -20°C. The expression assays were performed using samples from bone marrow and blood donors with normal hematological parameters (hematocrit, platelet, lymphocytes, monocytes, and granulocytes). The cDNA was synthesized from total RNA purified from 107 peripheral blood mononuclear cells isolated by density gradient centrifugation as described¹⁹.

Single nucleotide polymorphism (SNP) selection and genotyping. Nine candidate genes were selected on the basis of their function in innate host defense as dsRNA (*DDX58*, *IFIH1*, *TLR3*), ssRNA (*TLR7*, *TLR8*), dsDNA (*AIM2*, *IFI16*, *ZBP1*), and CpG DNA (*TLR9*) sensors. Tag SNP (tSNP) were selected across each locus from the designated set of common SNP genotyped in the CEU population (HapMap Project, Release 28, Phase II + III, US National Center for Biotechnology Information build 36 assembly, dbSNP b126; www.hapmap.org). The tSNP selection was done with pairwise $r^2 \geq 0.80$ and minor allele frequency ≥ 0.05 using the Haploview v4.0 software (www.broad.mit.edu/mpg/haploview/download.php)²⁰.

According to the above rules, 74 tSNP that permitted us to identify 216 SNP were selected (Supplementary Table 1, available online at jrheum.org). All 371 patients with BD and 854 controls were genotyped using the SEQUENOM iPLEX MassARRAY platform according to manufacturer's instructions (Sequenom). Primers for multiplex PCR and extended reactions were designed using the MassARRAY Assay Design Suite v1.0 software (www.sequenom.com). Primers design was not possible in 8 tSNP that were genotyped using TaqMan SNP Genotyping Assays (Applied Biosystems) in a LightCycler 480 (Roche; Supplementary Table 1). To verify interexperimental reproducibility and accuracy, 8% of the samples were duplicated. A 90% sample quality control rate and 90% SNP genotyping success rate was imposed on the analysis. Additionally, the

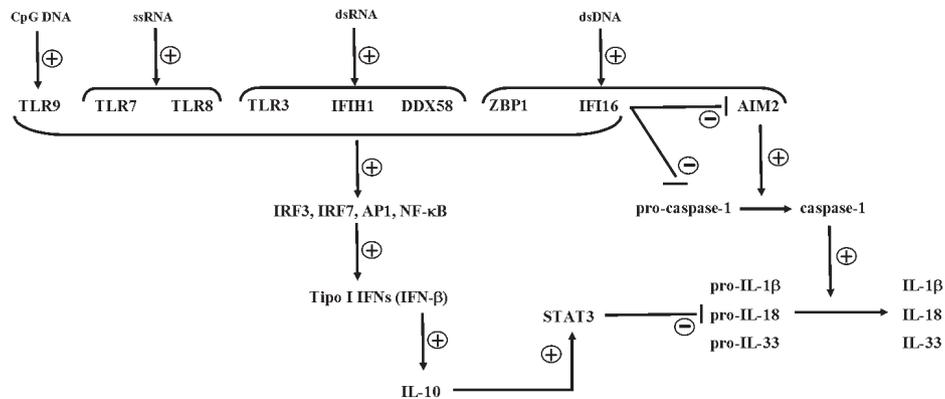


Figure 1. Pattern recognition receptors involved in the nucleic acid binding included in this study. TLR: Toll-like receptors; DDX58: DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (also known as retinoic acid inducible gene I); IFIH1: interferon (IFN) induced with helicase C domain 1 (or melanoma differentiation-associated factor 5); ZBP1: Z-DNA binding protein 1 (or DNA-dependent activator of IFN regulatory factor); IFI16: IFN- γ -inducible protein 16; AIM2: absent in melanoma 2; IRF3, IRF7: IFN-regulatory factor 3 and 7; AP-1: activator protein 1; NF- κ B: nuclear factor- κ B; IL: interleukin; STAT1: signal transducers and activators of transcription-1.

Table 1. Minor allelic frequencies of the SNP associated with Behçet disease in Spanish patients.

Gene SNP	MA	Controls, n = 854	Patients, n = 371	p	p _c	OR (95% CI)
IFI16						
rs7532207	T	0.087	0.118	0.017	0.047	1.41 (1.06–1.85)
rs6940	T	0.088	0.120	0.013	0.040	1.42 (1.07–1.87)
AIM2						
rs855873	A	0.105	0.072	0.010	0.031	0.66 (0.48–0.91)

P values > 0.05 were not significant. SNP: single-nucleotide polymorphism; MA: minor allele; p_c: 10,000-fold permutation testing.

rs6940 and rs855873 SNP were genotyped in a second group of 850 controls using TaqMan SNP Genotyping Assays.

Quantification of mRNA AIM2 and IFI16. The AIM2 and IFI16 mRNA expression assays were performed using 110 samples from healthy controls on a LightCycler 480 by TaqMan Gene Expression Assay (Applied Biosystems). The mRNA expression of AIM2 and IFI16 was correlated with the mRNA expression of the reference ABL gene²¹. The conditions of the assays and data analysis have been described¹⁹. Each sample was tested in triplicate, and only samples showing ABL Cp values between 24 and 30 and an SD of Cp < 0.3 were included in the final analysis (n = 102).

Allele-specific transcript quantification. IFI16 mRNA allele-specific transcript quantification (ASTQ) assays were performed by TaqMan SNP Genotyping Assays using 50 samples from healthy controls who were heterozygotes for the nonsynonymous SNP rs6940. The Cp values for both alleles labeled 1 with FAM (rs6940A) and the other with VIC (rs6940T) and were obtained for cDNA (target) and gDNA (reference), and a relative quantification model was performed²¹. To detect the presence of contaminating gDNA in the RNA samples, aliquots of total RNA were previously amplified. Those RNA samples that did not pass this test were not processed. The samples processed were tested in triplicate and only those samples showing gDNA Cp values between 24 and 30 with a Cp SD < 0.3 were included in the final analysis (n = 46).

Statistical analysis. Allele and haplotype frequency distributions between patients with BD and controls were compared with the chi-squared test. The OR with their corresponding 95% CI were calculated using OpenEpi v2.3 software online (www.openepi.com). Statistical significance was evaluated using a 10,000-fold permutation test (p_c) and those p_c values <

0.05 were considered statistically significant. The genetic model of inheritance was evaluated with Chaplin v1.2 software (www.genetics.emory.edu/labs/epstein/software/chaplin), using the likelihood ratio statistics for testing the global null hypothesis²² and the Akaike information criterion to determine the genetic model of inheritance²³. Results of relative IFI16 and AIM2 mRNA expression were compared using an unpaired t test with Welch's correction when the variances were not homogeneous (Levene's test < 0.05). To compare values obtained by ASTQ, a paired sample t test was used. Statistical analyses were performed using SPSS v18.0 software (SPSS Inc.), and p values < 0.05 were considered statistically significant.

RESULTS

Three hundred seventy-one patients and 854 controls were genotyped in tSNP of 9 genes involved in the recognition of nucleic acid molecules: 13 tSNP in IFI16, 2 in AIM2, 4 in IFI1, 2 in TLR9, 8 in TLR3, 16 in DDX58, 9 in ZBP1, 12 in TLR7, and 8 in TLR8, capturing 56, 2, 4, 3, 14, 72, 13, 29, and 23 SNP, respectively (Supplementary Table 1, available online at jrheum.org). The results of 4 of the 74 tSNP included were discarded because they had amplification failures or were not meeting quality criteria. In the remaining 70 tSNP, the concordance of the assigned genotypes in duplicated samples was > 98% and the study population was found in Hardy-Weinberg equilibrium (p > 0.05). Two tSNP located in the IFI16 locus (rs7532207 and

rs6940) and 1 tSNP located in the *AIM2* locus (rs855873) were significantly associated with BD (Table 1). The minor allele of the rs855873 (A) was protective (7.2 vs 10.5% in the control group, $p = 0.010$, OR 0.66, 95% CI 0.48–0.91), whereas both the minor alleles of rs6940 (T) and rs7532207 (T) were risk alleles (12.0 vs 8.8% in the control group, $p = 0.013$, OR 1.42, 95% CI 1.07–1.87; and 11.8 vs 8.7% in the control group, $p = 0.017$, OR 1.41, 95% CI 1.06–1.85, respectively). The rs7532207 was not included in subsequent assays because a strong linkage disequilibrium ($D' = 1$ and $r^2 = 0.988$) between rs7532207 and rs6940 was found in our population in both patient and control groups.

To confirm the association between the *IFI16/AIM2* locus and BD, the rs6940 and rs855873 were genotyped in 850 additional healthy controls. Samples from patients and controls of the first cohort that failed to amplify using SEQUENON were genotyped with TaqMan assays as the new control cohort. The total cohort was randomly divided into 2 groups: the cohort 1, which consisted of 187 patients and 854 controls, and cohort 2, which included 184 patients and 850 controls. After re-analyzing, both cohorts retained significant association with susceptibility to BD ($p = 0.046$ and 0.042 to rs6940, and $p = 0.039$ and 0.027 to rs855873; Table 2).

IFI16 and *AIM2* genes are located in a region spanning 70-kb on chromosome 1q25.2 (Figure 2) and they are transcribed in opposite directions. By performing the analysis of the haplotypes constructed by combination of the rs6940 and rs855873, 3 combinations with frequency > 0.005 were identified in our population (Table 3). One haplotype, “TG,” which was tagged by the rs6940T allele, was identified as a risk haplotype, whereas another haplotype, “AA,” tagged by the rs855873A, was identified as protective. Testing different genetic models of inheritance, we found that the recessive model could be excluded for both the risk and the protective haplotypes, whereas the multiplicative ($p = 0.016$ and $p = 0.0033$, respectively) and the dominant ($p = 0.019$ and $p = 0.0058$, respectively) models were the best fitting (Supplementary Table 2, available online at jrheum.org).

The mRNA expression levels of the *AIM2* and the *IFI16* in 3 kinds of samples obtained from healthy individuals were compared: those having the risk haplotype (1 or 2 copies), those with the protective haplotype (1 or 2 copies), and finally, those homozygous for the neutral haplotype (individuals AG/AG; Table 4). Statistically significant differences in *AIM2* mRNA expression levels were not found; nevertheless, samples with the risk haplotype had lower *IFI16* mRNA expression than samples with the protective haplotype (0.99 ± 0.29 vs 1.23 ± 0.50 , $p = 0.022$), whereas samples homozygous for the neutral haplotype had an intermediate level of expression (1.08 ± 0.46). To confirm that the risk haplotype has the lowest *IFI16* mRNA expression levels, the nonsynonymous SNP rs6940 (T723S) was used to perform an ASTQ assay. Heterozygous samples from healthy individuals showed statistically significant lower *IFI16* mRNA expression for the risk (T) than for the protective (A) allele ($p = 0.027$) with a ratio A/T < 1 (0.97 ± 0.08 ; Supplementary Figure 1, available online at jrheum.org).

DISCUSSION

In our study, including a total of 74 tSNP in 9 candidate genes encoding nucleic acid sensors, we identified the *IFI16-AIM2* gene cluster as a novel genetic susceptibility locus for BD in a Spanish population. In addition, our data suggest that the steady-state basal *IFI16* mRNA expression could be haplotype-dependent.

BD is included among disorders related to inflammasomes because serum levels of IL-1 β , IL-18, and IL-33 are elevated among patients^{24,25,26} who respond to treatment with neutralizing antibodies for IL-1 β ²⁷. Several inflammasome-dependent pathways have been involved in BD pathogenesis. Thus, recently, production of IL-1 β through an NLRP3-inflammasome-dependent pathway, which is initiated with interaction of TLR2/TLR4 with their ligands, has been implicated in the pathogenesis of the disease²⁸. Our results suggest that the *AIM2* inflammasome could also contribute to the pathogenesis of BD, mainly characterized by the presence of recurrent oral and genital ulceration. It

Table 2. Replication study in 2 tSNP in *IFI16*: rs6940 and rs855873. The total cohort, composed of 371 patients and 1704 controls, was randomly divided into 2 groups: cohort 1 (187 patients and 854 controls) and cohort 2 (184 patients and 850 controls).

SNP	Cohort 1					Cohort 2					Cohort 1 + 2							
	Controls, n = 854		Patients, n = 187		p	OR (95% CI)	Controls, n = 850		Patients, n = 184		p	OR (95% CI)	Controls, n = 1704		Patients, n = 371		p	OR (95% CI)
	AF	NA	AF	NA		AF	NA	AF	NA		AF	NA	AF	NA	AF	NA		
rs6940-T	0.089	153	0.123	46	0.046	1.42 (1.01–2.02)	0.088	150	0.122	45	0.042	1.44 (1.02–2.05)	0.088	303	0.122	91	0.0045	1.43 (1.12–1.83)
rs-6940-A	0.911	1555	0.877	328		0.912	1550	0.878	323		0.912	3105	0.878	651				
rs855873-A	0.107	184	0.072	27	0.039	0.64 (0.42–0.98)	0.106	180	0.068	25	0.027	0.61 (0.40–0.95)	0.107	364	0.070	52	0.0025	0.63 (0.46–0.85)
rs855873-G	0.893	1524	0.928	347		0.894	1520	0.932	343		0.893	3044	0.930	690				

tSNP: tag single nucleotide polymorphisms; AF: allelic frequency; NA: no. alleles.

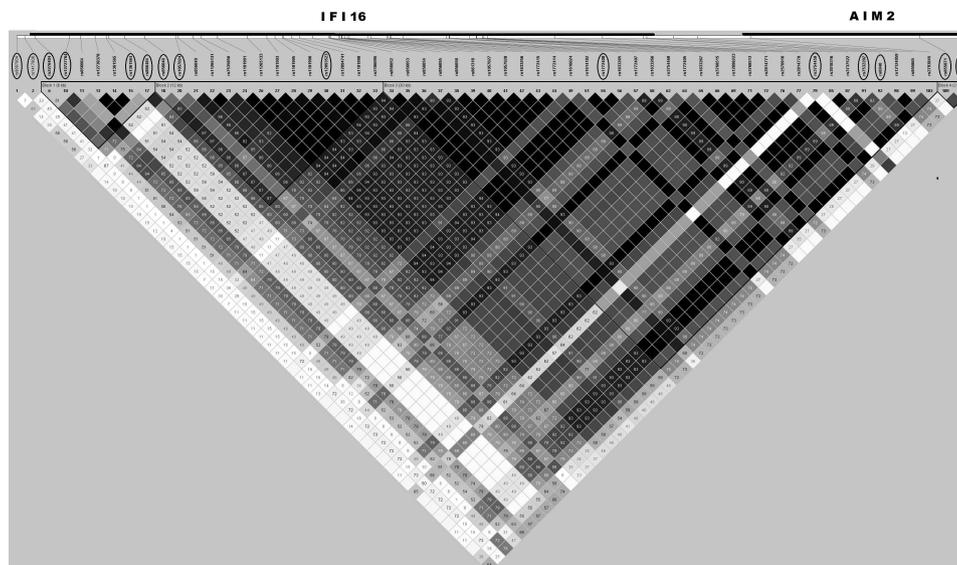


Figure 2. Haplotype blocks described in the region Chr1:157245220..157314336 of HapMap Project in the CEU population. The linkage disequilibrium (LD) solid spine approach was used to define the haplotype blocks. Standard color-coding was used for LD plots: white ($r^2 = 0$), shaded grey ($0 < r^2 < 1$), and black ($r^2 = 1$). Squares without a number indicate $D' = 1$. IFI16 and AIM2 loci are shown; marked rs numbers are the ones selected for this study.

Table 3. Allelic frequencies of the haplotypes constructed by combination of the 2 SNP associated with Behçet disease in Spanish patients.

	Haplotypes rs6940	rs855873	Controls, n = 854	Patients, n = 371	p	p_c	OR (95% CI)
N	A	G	0.809	0.810	ns		
R	T	G	0.086	0.117	0.015	0.045	1.41 (1.06–1.86)
P	A	A	0.103	0.070	0.009	0.026	0.65 (0.47–0.90)

SNP: single nucleotide polymorphisms; N: neutral haplotype; R: risk haplotype; P: protective haplotype; ns: not significant ($p > 0.05$); p_c : 10,000-fold permutation testing.

Table 4. AIM2 and IFI16 mRNA expression levels in samples from healthy controls with different phenotypes.

Phenotypes	N	AIM2, Mean \pm SD	p	IFI16, Mean \pm SD	p
Risk	31	1.04 \pm 0.57		0.99 \pm 0.29	
Protective	35	0.99 \pm 0.51	ns ¹	1.23 \pm 0.50	0.022 ¹
Neutral	36	0.87 \pm 0.47	ns ²	1.08 \pm 0.46	ns ²

¹Risk versus protective. ²Risk versus neutral. Risk phenotypes are individuals bearing 2 risk haplotypes (TG/TG) or 1 risk and 1 neutral (TG/AG). Protective phenotypes are individuals bearing 2 protective haplotypes (AA/AA) or 1 protective and 1 neutral haplotype (AA/AG). Neutral phenotypes are individuals homozygous for the neutral haplotype (AG/AG). ns: not significant.

has been described as a strong upregulation of AIM2 protein expression in the epidermis of patients with several acute and chronic inflammatory skin disorders such as psoriasis, atopic dermatitis, venous ulcers, contact dermatitis, and in studies on wound healing^{29,30}. Possibly, AIM2 upregulation is a first line of defense against invading pathogens after skin barrier disruption. Nevertheless, this beneficial effect during wound healing could establish a chronic inflam-

matory injury in the skin when barrier impairment is prolonged because of activation induced by the IL-1 β AIM2³¹.

IFI16 and AIM2 are cytosolic sensors for dsDNA and both are members of the IFN-inducible p200 family (IFI200), which is also referred to as ALR³². IFI16 and AIM2 proteins have different effects because of their different pyrin domains (PYD), which allow protein-protein

interaction (Figure 1). The IFI16 protein, after recruiting STING (stimulator of interferon genes) through its PYD domain, stimulates the expression of IFN- β by activation of IRF3 and NF- κ B¹². Finally, IFN- β inhibits the production of IL-1 β by inflammasomes through a mechanism that is dependent on signal transducers and activators of transcription-1³³. For its part, the AIM2 protein, through its PYD domain, interacts with apoptosis-associated speck-like protein containing a CARD domain, which activates caspase-1 through its CARD domain¹³ and leads to inflammation by the cleavage of inflammatory cytokine proforms, such as IL-1 β , IL-18, and IL-33^{15,16,17}. Therefore, IFI16 has antiinflammatory effects, whereas AIM2 has proinflammatory effects. Moreover, IFI16 downregulates the expression of AIM2 and pro-caspase-1 and as a consequence, it suppresses the activation of caspase-1 by the AIM2 and NLRP3 inflammasomes¹⁴. Further, AIM2 and IFI16 can form heterodimers in the cytoplasm, and by this mechanism, IFI16 also downregulates the expression of an AIM2 inflammasome.

The IFI16/AIM2 ratio in the cytoplasm contributes to the balance between the production of type I IFN and IL-1 β ¹⁴. A study involving young, old, and senescent human diploid fibroblasts supports the theory that expression of IFI16 and AIM2 proteins is inversely correlated³⁴. Our data suggested that the steady-state basal *IFI16* mRNA expression could be haplotype-dependent because the risk haplotype has the lowest *IFI16* mRNA levels. These results were confirmed by performing ASTQ assays, which are not affected by confounding factors because mRNA levels for each allele are measured in the same sample of RNA and therefore differences in the mRNA expression levels for both alleles are a consequence of differences within the cis-acting regulatory elements. The analysis of expression was not performed in patients, to avoid problems related to different treatments (biologics and monoclonal therapy), which could modify the steady-state basal expression of the molecules under study.

Genome-wide association studies (GWAS) have identified several loci of susceptibility to BD outside the *HLA* region in different populations^{7,8,35,36,37,38}; nevertheless, association with the *IFI16* gene has not been described in any other population. Failure to identify this association in GWAS could be due to various reasons. The high-throughput platforms used in early GWAS for BD did not include the tSNP that we found associated in our study or other tSNP with $r^2 = 1$ with the rs6940 and rs855873^{7,8,35}. Although these tSNP are included in the high-throughput platforms of Affimetrix used in more recent studies^{36,37}, the sample size of the cohorts included was not large enough to detect the association with the threshold p-value levels used in GWAS.

Our study has limitations. The sample size allows only slightly significant results, and the low prevalence of the

disease in Spain does not permit recruiting many more patients. Therefore, these data need to be replicated in other populations with a higher prevalence of BD. However, it is also necessary to note that association of some non-*HLA* loci such as *IL-10* and *IL23R-IL12RB* with the disease has been described in several populations, whereas other loci (such as *KIAA1529*, *CPVL*, *LOC100129342*, *UBASH3B*, *UBAC2*, *ERAP1*, *STAT4*, and *GIMAP*) have been found only in a particular ethnic group^{7,8,37,38,39}.

Our results suggest a relationship between the IFI16, a mediator of the AIM2 inflammasome-dependent pathway, and susceptibility to BD. This association could be explained by steady-state basal *IFI16* mRNA expression.

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ONLINE SUPPLEMENT

Supplementary data for this article are available online at jrheum.org.

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