

# KIR3DL1/S1 Allotypes Contribute Differentially to the Development of Behçet Disease

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Behçet disease is a chronic, relapsing-remitting autoinflammatory syndrome with a strong *HLA-B\*51* association. In this paper, we describe a human cohort of 267 individuals with Behçet disease and 445 matched controls from a tertiary referral center in the U.K. *HLA-B\*51* was confirmed as a genetic risk factor in this group ( $p = 0.0006$ , Bonferroni–Dunn correction for multiple testing [ $P_c$ ] = 0.0192, odds ratio [OR] 1.92, 95% confidence interval [CI] 1.33–2.76). KIR3DL1/S1 allele-level analysis indicated that low-expressing KIR3DL1/S1 alleles in combination with KIR3DS1 increased the risk of developing Behçet disease (KIR3DL1<sup>LOW</sup>/KIR3DS1:  $p = 0.0004$ ,  $P_c = 0.0040$ , OR 2.47, 95% CI 1.43–4.25), whereas high-expressing KIR3DL1/S1 alleles in combination with a null-expressing KIR3DL1 reduced the risk of disease (KIR3DL1<sup>HIGH</sup>/KIR3DL1<sup>NULL</sup>:  $p = 0.0035$ ,  $P_c = 0.0350$ , OR 0.53, 95% CI 0.33–0.87). Behçet disease can manifest as a purely mucocutaneous disease or can involve other organ systems such as the eyes. In the U.K. cohort studied in this study, KIR3DL1<sup>LOW</sup>/KIR3DS1 increased the risk of ophthalmic disease ( $p = 1.2 \times 10^{-5}$ , OR 3.92, 95% CI 2.06–7.47), whereas KIR3DL1<sup>HIGH</sup>/KIR3DL1<sup>NULL</sup> reduced the risk of having purely mucocutaneous disease ( $p = 0.0048$ , OR 0.45, 95% CI 0.25–0.81). To our knowledge, this is the first analysis of KIR3DL1/S1 allelic variation in Behçet disease and may provide insight into the pathogenic role of *HLA-B\*51* and its interaction with KIR3DL1/S1. *The Journal of Immunology*, 2019, 203: 1629–1635.

**B**ehçet disease (BD) is a chronic autoinflammatory, relapsing-remitting, multisystemic syndrome. It is characterized by orogenital ulcers, papulopustular and erythema nodosum-like skin lesions, uveitis, retinal vasculitis, thrombophlebitis, arterial aneurysms, and arthritis. BD is found primarily along the ancient Silk Route from the Mediterranean Basin across Asia to Japan, but cohorts have also been reported in Northern Europe, North America, and South America (1). The

prevalence of BD across the Middle East and Asia is 20–400 per 100,000 (2–4). Recent data from the U.K. BD Centres of Excellence in 2017 suggest the incidence in the United Kingdom is likely to be at least 2 per 100,000 (H. Petrushkin and F. Fortune, unpublished observations), an increase on previous estimates (5), suggesting the impact of this disease is underrecognized in individuals of European extraction.

BD has a genetic basis, with the strongest association loci residing within the *HLA-B* region on chromosome 6 and with *HLA-B\*51* in particular. Evidence for this association was first described in 1982, when Ohno et al. observed that HLA-Bw51 (now *HLA-B\*51*) was associated with BD in Japanese patients. Recent meta-analyses have shown *HLA-B\*51* carriage increases the risk of BD by a factor of six. Eight genome-wide association studies (GWAS) investigating BD have been carried out that support single nucleotide polymorphisms in noncoding regions located between the *HLA-A–HLA-F* loci, with considerable interstudy heterogeneity regarding other reported associations (6, 7). These GWAS have mainly been carried out in patients from regions of the world where BD is most prevalent (Turkey, Japan, China, Korea, Italy, and Iran), although in 2015, a mixed population of patients with BD from the Netherlands was reported (6, 8–14).

To date, the mechanistic association between *HLA-B\*51* and disease pathogenesis remains unclear. HLA class I molecules present Ag to CD8 cytotoxic T cells; however, there is little evidence for a role of these cells in BD. Certain HLA class I molecules are also ligands for the killer Ig-like receptor (KIR) molecules expressed on NK cells. KIR3DL1, a polymorphic inhibitory receptor, interacts with *HLA-B\*51* via the Bw4 epitope, a region spanning residues 77–83 on the  $\alpha 1$  helix of certain HLA-A and -B allotypes (15, 16). KIR3DL1 is an inhibitory receptor encoded by multiple alleles of the *KIR3DL1/S1* gene, whereas KIR3DS1 is a conserved, activating receptor encoded at the same locus. KIR3DS1 is primarily thought to interact with HLA-F (17–20). The *KIR* gene cluster has only recently been defined at

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Abbreviations used in this article: BD, Behçet disease; CI, confidence interval; ERAP-1, endoplasmic reticulum aminopeptidase-1; GWAS, genome-wide association study; HC, healthy control; KIR, killer Ig-like receptor; MC, mucocutaneous; OR, odds ratio;  $P_c$ , Bonferroni–Dunn correction for multiple testing.

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a high resolution, and disease-specific associations with KIR allotypes are limited (21–24).

Previously, Middleton et al. (25) examined Turkish patients with BD for the presence/absence of *KIR3DL1/SL* but found no association after controlling for the effects of *HLA-B\*51*. Erer et al. (26) studied a large, carefully compiled dataset of 1799 Turkish BD patients and reported an association between *KIR3DS1* and ocular disease, but no effect of *KIR3DL1/SL* overall on disease risk. More recently, Mohammad-Ebrahim et al. (27) examined 397 Iranian patients with BD but found no specific *KIR* gene to be associated with BD. At present, there are no studies that analyze *KIR3DL1/SL* allelic variation or their effect in BD. In this study, we set out to characterize *HLA-A*, *-B*, and *KIR3DL1/SL* genotype frequencies in a cohort of BD patients from the United Kingdom.

## Materials and Methods

### Patients

Two hundred sixty-seven individuals with BD and four hundred forty-five demographically matched healthy controls (HC) were recruited from the BD Centre of Excellence at the Royal London Hospital of Bart's Health National Health Service Trust (London, U.K.). All patients gave written consent, and the project was approved by the local research ethics service of the City and East London (P/03/122). The study conformed to the Declaration of Helsinki. All patients fulfilled the Behcet Disease International Study Group diagnostic criteria (28). Samples were collected between 2013 and 2015. Individuals in the HC group were not related to individuals in the BD group, and every effort was made to match the two groups for sex and ethnicity. To avoid recruiting controls with undiagnosed BD, all HC were over 40 y old because it is uncommon for BD to manifest past the fourth decade.

### Genotyping

Blood samples were collected in 10 ml EDTA tubes, and DNA was extracted with QIAamp DNA Blood Maxi Kits (QIAGEN, Hilden, Germany). DNA was eluted in AE buffer and stored at  $-80^{\circ}\text{C}$  until use. *HLA-B* and *KIR3DL1/SL* genes were sequenced using a Fluidigm library preparation (Fluidigm, South San Francisco, CA). Amplicons were designed to span the coding DNA sequence of *HLA-B* and *KIR3DL1/SL* (primers available upon request). Sequencing was carried out using a MiSeq platform (Illumina, San Diego, CA) at the Bart's and the London Genome Centre (Bart's and the London Medical School, Charterhouse Square, London, U.K.). *HLA-B* alleles were assigned using NGSengine software (GenDx, Utrecht, the Netherlands), and *KIR3DL1/SL* alleles were assigned using the Pushing Immunogenetics to the Next Generation pipeline (29).

### HLA-A typing

*HLA-A* genotyping was carried out using sequence-specific oligonucleotide probes (Immucor, Peachtree Corners, GA) on a Luminex platform (Luminex, Austin, TX) at the Viapath Clinical Transplantation Laboratory at Guys Hospital (London, U.K.).

### Statistical analysis

Probabilities for categorical data were calculated using a two-tailed Fisher exact test. Nonparametric, ordinal data were analyzed with a Mann-Whitney *U* test. Differences between groups were calculated using a two-way ANOVA. The Bonferroni–Dunn correction for multiple testing (*P<sub>c</sub>*) was applied (GraphPad Prism 7, GraphPad Software, La Jolla, CA). Where the frequency of an outcome was 0, the Haldane correction was used to estimate the odds ratio (OR) and 95% confidence intervals (CI). The magnitude of the effect was estimated using the OR and 95% CI.

For 80% power with an  $\alpha = 0.05$ , assuming a 1:2 case/control recruitment, we aimed to collect at least 199 cases and 398 controls. This was based on the population prevalence of *KIR3DL1\*001* in the United Kingdom of 15% (30). As the exact *KIR3DL1/SL* allele frequencies within a United Kingdom-based BD cohort have not been explored, we collected as many samples as possible to allow for patients withdrawing from the study and technical problems with samples.

A *p* value  $\leq 0.05$  was considered to be statistically significant but only in the context of a 95% CI that did not cross 1.00. If *p* < 0.05 but *P<sub>c</sub>* > 0.05 and the 95% OR did not cross 1, the result was considered relevant for discussion.

## Results

The group demographics, including self-reported ethnicity, are presented in Table I. There are no significant differences between the sex or ethnicity of the cases and controls. The control group was, on average, 13 y older than the BD group. The population analyzed in this paper is of mixed ethnicity with 67.4% being of self-reported Northern European ancestry. Clinical manifestations along with the ethnicity of the patient groups are given in Fig. 1. After quality control, *HLA-A* genotypes were obtained for 260 (97.38%) cases and 426 (95.73%) controls, *HLA-B* genotypes were obtained for 266 (99.63%) cases and 443 (99.55%) controls, and *KIR3DL1/SL* genotypes were obtained for 256 (95.88%) cases and 433 (97.30%) controls.

### Association of *HLA-B\*51* with BD is replicated in the U.K. cohort

*HLA-B\*51* was associated with BD (*p* = 0.0006, *P<sub>c</sub>* = 0.0192, OR 1.92, 95% CI 1.33–2.76). A smaller effect was also identified for *HLA-B\*08* (*p* = 0.0238, *P<sub>c</sub>* = NS, OR 1.49, 95% CI 1.06–2.11), *HLA-A\*25* (*p* = 0.0121, *P<sub>c</sub>* = NS, OR 2.51, 95% CI 1.17–5.41), and *HLA-A\*26* (*p* = 0.0291, *P<sub>c</sub>* = NS, OR 1.76, 95% CI 1.00–3.10). A suggestion of protective effects was found for *HLA-B\*58* (*p* = 0.0248, *P<sub>c</sub>* = NS, OR 0.21, 95% CI 0.05–0.90), and *HLA-A\*33* (*p* = 0.0131, *P<sub>c</sub>* = NS, OR 0.45, 95% CI 0.22–0.92) (Table II).

The HLA class I data were analyzed for the presence/absence of Bw4, and the gene dosage of Bw4 encoding alleles was calculated (i.e., an individual may have a maximum of four Bw4 encoding alleles if both alleles of *HLA-A* and *-B* encode HLA allotypes with the Bw4 epitope such as *HLA-A\*23/A\*24* and *HLA-B\*51/B\*44*). The results indicate that there was no disease association between the presence/absence or gene dosage of Bw4 encoding alleles and BD (data not shown).

### *KIR3DL1/SL* “functional genotypes” are associated with BD

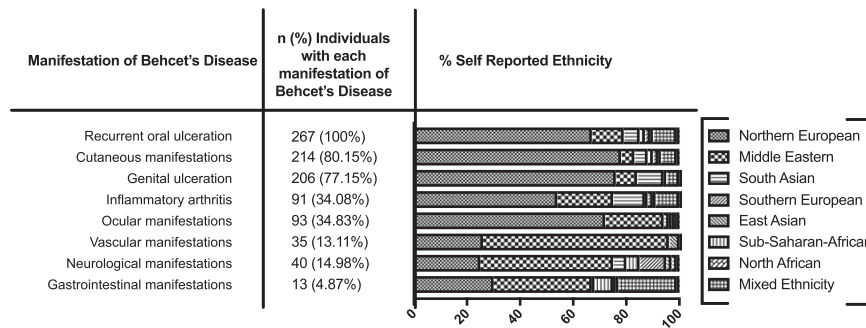
Having established that *HLA-B\*51* conferred a risk of BD in this cohort of patients, we investigated the effects of *KIR3DL1/SL* allelic variation. *KIR3DL1* was present in 96% of individuals in both groups. There was no difference in the frequency of *KIR3DL1/SL* heterozygotes between the groups and no association between the *KIR3DL1/SL* presence/absence, and BD was identified. Nor was there any departure from Hardy–Weinberg

Table I. Patient and HC demographics

	Control (n)	BD (n)	<i>p</i> Value
Participants	445	267	—
Sex			
Male	191	108	NS
Female	254	159	NS
Age	54 (IQ-range 54–58)	41 (IQ-range 36–43)	<i>p</i> < 0.0001
Ethnicity (self-reported)			
Northern European	302	180	NS
Middle Eastern	39	31	NS
South Asian	40	15	NS
Sub-Saharan-African	2	6	NS
Southern European	13	6	NS
East Asian	12	4	NS
Mixed ethnicity	31	23	NS
North African	6	2	NS

The cases and controls were matched for gender and ethnicity. The control group was selected to be over 50 y of age as BD usually manifests in the first five decades of life. This was done to ensure no individuals with an unmasked autoimmune disease were incorrectly labeled as healthy.

IQ, interquartile.



**FIGURE 1.** The diagnosis of BD was made in a tertiary referral center by a multidisciplinary team, with experts from the following fields: oral medicine, rheumatology, ophthalmology, neurology, immunology, and psychology. Percentage of the self-reported ethnicity can be seen next to each manifestation of BD. The majority of patients presenting with MC disease, arthritis, and ocular disease were of Northern European ethnicity, in keeping with the overall makeup of the cohort. Of note, increased percentages of Middle Eastern patients had vascular, neurologic, and gastrointestinal manifestations.

Equilibrium between the BD and HC groups (Supplemental Table I). We then analyzed our disease cohorts to ascertain whether any individual *KIR3DL1/S1* allele increased or decreased the risk of developing BD (Supplemental Table III); *KIR3DL1\*005* and *KIR3DS1\*013* were found to contribute toward the risk of disease, but the association did not survive correction for multiple testing.

We grouped *KIR3DL1/S1* allotypes by their expression phenotype based on previously published data (31–36), to create functional genotypes. For those allotypes in which functional data were not available, we assigned a presumptive expression phenotype based on the nearest *KIR3DL1/S1* allotypes for which there is data (Supplemental Table II). We found no association between high-expressing *KIR3DL1* (*KIR3DL1<sup>HIGH</sup>*), low-expressing *KIR3DL1* (*KIR3DL1<sup>LOW</sup>*), *KIR3DL1* retained intracellularly (*KIR3DL1<sup>NULL</sup>*), or the activating allele, *KIR3DS1* (data not shown). In contrast, we found the combination of *KIR3DL1<sup>LOW</sup>/KIR3DS1* was associated with BD ( $p = 0.0004$ ,  $P_c = 0.0040$ , OR 2.47, 95% CI 1.43–4.25), whereas the combination of *KIR3DL1<sup>HIGH</sup>/KIR3DL1<sup>NULL</sup>* was associated with protection from disease ( $p = 0.0035$ ,  $P_c = 0.0350$ , OR 0.53, 95% CI 0.33–0.87) (Table III). The increased and decreased risk conferred by *KIR3DL1<sup>LOW</sup>/KIR3DS1* and *KIR3DL1<sup>HIGH</sup>/KIR3DL1<sup>NULL</sup>*, respectively, remained when we removed those alleles without direct evidence of their expression level (Supplemental Table IV). These results indicate that the *KIR3DL1<sup>LOW</sup>/KIR3DS1* functional genotype is associated with the risk of developing BD, whereas the *KIR3DL1<sup>HIGH</sup>/KIR3DL1<sup>NULL</sup>* functional genotype is associated with protection from BD.

We then analyzed the allelic composition of the implicated functional genotypes. There were two genotypes associated with BD: *KIR3DL1\*0050101<sup>LOW</sup>/KIR3DS1\*0130101* ( $p = 0.0210$ , OR 1.91, 95% CI 1.07–3.43) and *KIR3DL1\*0070101<sup>LOW</sup>/KIR3DS1\*0130101*

( $p = 0.0008$ , OR 15.22, 95% CI 1.92–120.87). Conversely, *KIR3DL1\*0010101<sup>HIGH</sup>/KIR3DL1\*00401<sup>NULL</sup>* ( $p = 0.0172$ , OR 0.36, 95% CI 0.15–0.89) was found to be protective (Table IV).

To examine whether the *KIR3DL1/S1* functional genotypes conferring risk and protection are associated with BD or merely the presence of *Bw4/HLA-B\*51*, we repeated the above analysis, controlling for these variables. We found the effect of possessing a *KIR3DL1<sup>LOW</sup>/KIR3DS1* risk genotype to be significant in all groups except the *HLA-B\*51<sup>+</sup>* individuals. The protective effects of the *KIR3DL1<sup>HIGH</sup>/KIR3DL1<sup>NULL</sup>* genotype were only significant in individuals possessing *Bw4* (Fig. 2).

*Differential associations between KIR3DL1/S1 functional genotypes and clinical manifestations of BD*

The BD population was then split into two subgroups: those with purely mucocutaneous (MC) disease ( $n = 166$ ) and those with ophthalmic involvement ( $n = 91$ ). The MC and ophthalmic groups did not significantly differ with regard to their ethnic makeup ( $p = \text{NS}$ ) (Fig. 1). The two groups were then compared with the HC group. *HLA-B\*51* remained associated with BD in both groups (MC group:  $p = 0.0009$ , OR 4.14, 95% CI 2.72–6.32; ophthalmic group:  $p = 0.0367$ , OR 1.75, 95% CI 1.05–2.92), and there was no difference between the groups with regard to *KIR3DL1/S1* presence/absence (data not shown). We were unable to replicate the association between an increase in *KIR3DS1* alleles and ophthalmic involvement as was previously reported (26).

Examining *KIR3DL1/S1* allele combinations in the MC group revealed an increase in *KIR3DL1\*00701/KIR3DS1\*013* compared with HCs ( $p = 0.0071$ , OR 13.04, 95% CI 1.51–112.49). Despite this, none of the functional genotypes were associated with MC disease. Conversely, no specific combination of *KIR3DL1/S1* alleles conferred protection to MC disease, but the functional

Table II. *HLA-A* and *-B* associations in the U.K. cohort

HLA Type	U.K. Cohort (HC $n = 445$ , BD $n = 267$ )							
	HC (n)	HC (%)	BD (n)	BD (%)	<i>p</i> Value	<i>P<sub>c</sub></i>	OR	95% CI
<b>Increased risk</b>								
<i>HLA-B*51</i>	62	6.97	67	12.55	<b>0.0006</b>	<b>0.019</b>	1.92	1.33–2.76
<i>HLA-B*08</i>	78	8.76	67	12.55	<b>0.0238</b>	NS	1.49	1.06–2.11
<i>HLA-A*25</i>	11	1.2	17	3.1	<b>0.0121</b>	NS	2.51	1.17–5.41
<i>HLA-A*26</i>	24	3.2	26	5.1	<b>0.0291</b>	NS	1.76	1.00–3.10
<b>Reduced risk</b>								
<i>HLA-B*58</i>	16	1.80	2	0.37	<b>0.0248</b>	NS	0.21	0.05–0.90
<i>HLA-A*33</i>	36	4.3	10	2.3	<b>0.0131</b>	NS	0.45	0.22–0.92

Those in bold are significant ( $p < 0.05$ ) results in which the 95% CI does not cross 1.

Table III. The effects of the functional genotype *KIR3DL1/S1* combinations

<i>KIR3DL1/S1</i> allele		HC n=433, BD n=256							
Allele 1	Allele 2	HC (n)	HC (%)	BD (n)	BD (%)	P	Pc	OR	95% CI
<i>3DL1<sup>HIGH</sup></i>	<i>3DL1<sup>HIGH</sup></i>	85	19.63	60	23.44	NS	NS	1.19	0.82-1.74
<i>3DL1<sup>HIGH</sup></i>	<i>3DL1<sup>LOW</sup></i>	71	16.40	36	14.06	NS	NS	0.86	0.56-1.32
<i>3DL1<sup>HIGH</sup></i>	<i>3DL1<sup>NULL</sup></i>	76	17.55	24	9.38	<b>0.0035</b>	<b>0.0350</b>	0.53	0.33-0.87
<i>3DL1<sup>HIGH</sup></i>	<i>3DS1</i>	83	19.17	39	15.23	NS	NS	0.79	0.52-1.21
<i>3DL1<sup>LOW</sup></i>	<i>3DL1<sup>LOW</sup></i>	17	3.93	7	2.73	NS	NS	0.70	0.28-1.7
<i>3DL1<sup>LOW</sup></i>	<i>3DL1<sup>NULL</sup></i>	23	5.31	16	6.26	NS	NS	1.18	0.61-2.27
<i>3DL1<sup>LOW</sup></i>	<i>3DS1</i>	24	5.54	35	13.67	<b>0.0004</b>	<b>0.0040</b>	2.47	1.43-4.25
<i>3DL1<sup>NULL</sup></i>	<i>3DL1<sup>NULL</sup></i>	10	2.31	7	2.73	NS	NS	1.18	0.45-3.15
<i>3DL1<sup>NULL</sup></i>	<i>3DS1</i>	30	6.93	23	8.98	NS	NS	1.30	0.74-2.29
<i>3DS1</i>	<i>3DS1</i>	14	3.23	9	3.52	NS	NS	1.09	0.46-2.55

*KIR3DL1<sup>HIGH</sup>*-*KIR3DL1* alleles are highly expressed and confer strong inhibition to cell-mediated lysis. *KIR3DL1<sup>LOW</sup>*-*KIR3DL1* alleles are expressed at a low level and confer weak inhibition to cell-mediated lysis. *KIR3DL1<sup>NULL</sup>*-*KIR3DL1*\*004 is mostly retained intracellularly and has minimal inhibitory potential. *KIR3DS1* activates *KIR* allele. Those in bold signify significant ( $p < 0.05$ ) results in which the 95% CI does not cross 1. Red indicates an inhibitory *KIR3DL1* allotype expressed at high levels on the cell surface, pink indicates an inhibitory *KIR3DL1* allotype expressed at low levels on the cell surface, yellow indicates a null *KIR3DL1* allotype not expressed on the cell surface, and green indicates an activating *KIR3DL1* allotype.

genotype *KIR3DL1<sup>HIGH</sup>/KIR3DL1<sup>NULL</sup>* reduced the risk of developing purely MC disease ( $p = 0.0048$ , OR 0.45, 95% CI 0.25–0.81).

The ophthalmic group had an increased frequency of *KIR3DL1\*00501/KIR3DS1\*013* ( $p = 0.0003$ , OR 3.27, 95% CI 1.65–6.48) and *KIR3DL1\*00701/KIR3DS1\*013* ( $p = 0.0039$ , OR 18.83, 95% CI 2.08–170.47) compared with controls. Of note, the functional genotype *KIR3DL1<sup>LOW</sup>/KIR3DS1* conferred significant risk ( $p = 1.2 \times 10^{-5}$ , OR 3.92, 95% CI 2.06–7.47) in these individuals. There were no protective effects observed in this group (Fig. 3).

## Discussion

*KIR* expression is variable on individual NK cells, and this can have a major influence on the response to immune challenge (37). We have examined the risk alleles associated with BD in a large cohort of patients in the United Kingdom, the majority of whom are of Northern European heritage. As expected, the strongest risk association was found with *HLA-B\*51* with weaker associations from *HLA-B\*08*, *HLA-A\*25*, and *HLA-A\*26*. Protective effects were identified from *HLA-B\*58* and *HLA-A\*33*, confirming previous studies in European and Korean patients (38, 39).

As stated, *KIR3DL1/S1* recognizes the Bw4 epitope at the C terminus of the  $\alpha$ -chains forming the peptide binding groove in a subset of MHC class I molecules. As such, polymorphisms within *KIR3DL1/S1* can alter the strength of binding and thus the inhibitory potential of the *KIR*-HLA interaction.

Because of small sample numbers, some specific allele associations did not remain significant after multiple testing [both *KIR3DL1\*00501* and *KIR3DS1\*01301* showed a trend toward an association with BD (Supplemental Table III)] but nevertheless

deserve further attention with a wider cohort and functional studies.

The functional genotype results show an association between a risk genotype made up of a *KIR3DL1<sup>LOW</sup>* allele (*\*00501* or *\*00701*) and the activating allele *KIR3DS1\*013* (*KIR3DL1<sup>LOW</sup>/KIR3DS1*) and protective effects from the genotype made up of *KIR3DL1\*0010101* and *\*00401* (*KIR3DL1<sup>HIGH</sup>/KIR3DL1<sup>NULL</sup>*). In 2018, Martin et al. (40) described a single variant in *KIR3DL1* that significantly modified progression of HIV-1 due to the protective response from HLA-B\*57, supporting the concept of *KIR/HLA* influencing disease outcome.

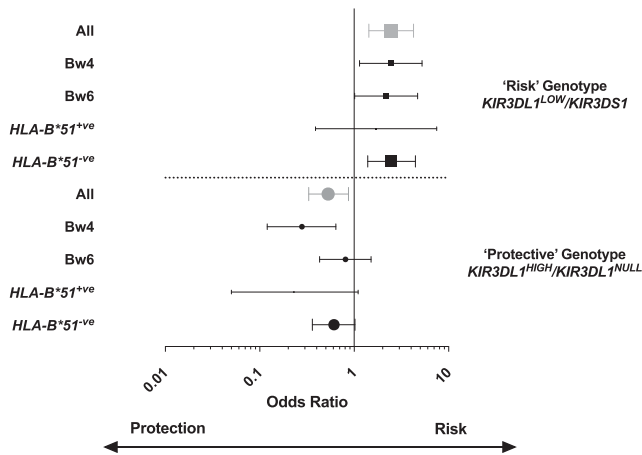
Despite many structural similarities between *KIR3DL1* and *KIR3DS1*, it remains contentious as to whether Bw4 is a ligand for *KIR3DS1*. There has been one report of *KIR3DS1* binding HLA-B\*57 (41) in the context of HIV-1 infection. More recently, HLA-F was identified as a high-affinity ligand for *KIR3DS1* (18). HLA-F neither binds peptide nor requires  $\beta$ 2-microglobulin to fold stably. It is upregulated during cell stress (42) and has also been implicated in NK cell education (19). HLA-F also binds allotypes of *KIR3DL1* but with a lower affinity. In BD, *KIR3DS1* may recognize HLA-F that has been upregulated on lymphocytes because of cell stress, leading to a persistent proinflammatory state, which could be exacerbated by an immune “trigger” such as a viral infection or mechanical trauma, downregulation of cell-surface HLA, and increased cell-mediated cytotoxicity. This, in conjunction with a *KIR3DL1<sup>LOW</sup>* allotype, may result in NK cells that are more likely to degranulate than those without an inhibitory *KIR3DL1* or indeed an activating receptor. *KIR3DS1* has also been linked to a number of other diseases without a Bw4 association (43–45), suggesting that the effects of *KIR3DS1* can be

Table IV. *KIR3DL1* alleles that make up the risk and protective functional genotypes

<i>KIR3DL1/S1</i> allele	UK Cohort (HC n=445, BD n=267)							
	HC (n)	HC (%)	BD (n)	BD (%)	P	Pc	OR	95% CI
Increased Risk								
<i>3DL1*0050101</i>	23	5.16	26	9.73	<b>0.0210</b>	NS	1.91	1.07-3.43
<i>3DL1*0070101</i>	1	0.23	9	3.37	<b>0.0008</b>	<b>0.0307</b>	15.22	1.92-120.87
Decreased Risk								
<i>3DL1*0010101</i>	28	6.29	6	2.24	<b>0.0172</b>	NS	0.36	0.15-0.89

A combination of the weakly expressed *KIR3DL1\*005* and *007* were the *KIR3DL1* alleles implicated in the risk genotype in conjunction with *KIR3DS1\*013*. The protective genotype was made up of highly expressed *KIR3DL1\*001* and *KIR3DL1\*004*, a null-expressing allele. Those in bold signify significant ( $p < 0.05$ ) results in which the 95% CI does not cross 1. Red indicates an inhibitory *KIR3DL1* allotype expressed at high levels on the cell surface, pink indicates an inhibitory *KIR3DL1* allotype expressed at low levels on the cell surface, yellow indicates a null *KIR3DL1* allotype not expressed on the cell surface, and green indicates an activating *KIR3DL1* allotype.





**FIGURE 2.** Square markers represent “risk” genotypes, and circular markers represent “protective” genotypes. The size of the markers are proportional to the size of the subgroups. Points above the dotted line represent the OR and 95% CIs for  $KIR3DL1^{LOW}/KIR3DS1$  risk group. Data are shown (in descending order) for all individuals and then only those that possess Bw4 (one or two copies), Bw6 (two copies), and *HLA-B\*51* (one or two copies) and those that lack *HLA-B\*51*. The points below the dotted line represent the OR and 95% CIs for the protective genotype  $KIR3DL1^{HIGH}/KIR3DL1^{NULL}$  with the same data groups.

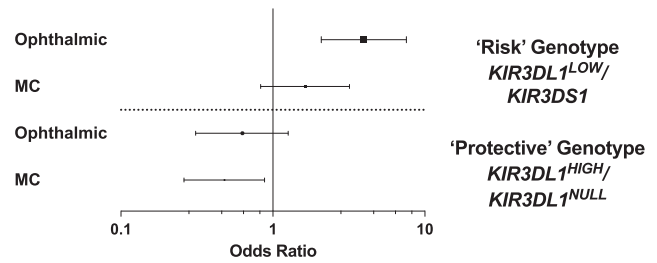
independent of Bw4. Similarly, the  $KIR3DL1^{LOW}/KIR3DS1$  functional genotype described in this study was found to be significant in the Bw6 subgroup in addition to the Bw4 subgroup.

The protective  $KIR3DL1^{HIGH}/KIR3DL1^{NULL}$  genotype has no activating KIR3D and thus relies on other activating receptors to initiate degranulation. Why  $KIR3DL1^{HIGH}/KIR3DL1^{NULL}$  should result in protection from disease whereas  $KIR3DL1^{HIGH}/KIR3DL1^{LOW/HIGH}$  does not remains unclear. It may be that a specific degree of KIR3DL1 surface tuning is required to provide protective effects and that only  $KIR3DL1*004$  is able to provide this. It is also possible that only a small fraction of total  $KIR3DL1*004$  is required to interact with Bw4 to lead to downstream effects as previously suggested by the protective effects of  $KIR3DL1*004$  in HIV progression (46).

The role of  $KIR3DL1*004$  in disease is of current interest. Whereas  $KIR3DL1*004$  was shown to be retained intracellularly and not contribute to the inhibitory response (47), Taner et al. (46) demonstrated that a small portion is correctly folded and cell surface expressed. There is evidence to suggest that possession of Bw4 and  $KIR3DL1*004$  slows progression of HIV infection to AIDS (48). Whether  $KIR3DL1*004$  can be considered ‘null’ in the context of disease association is a subject for further investigation.

Given the number of genetic risk factors uncovered by recent GWAS, there are probably many different pathways, each with small effects, that lead to the development of BD. The HLA–KIR interaction may be a feasible biological explanation, but it fails to explain the role of  $KIR3DL1/S1$  in Bw4<sup>-</sup> patients with BD. Whether there is an increased role for CD8 T cells–KIR interactions in these individuals remains to be seen (49).

The putative hypothesis driving this project was that  $KIR3DL1$  allotypes interact with Bw4 (and therefore *HLA-B\*51*) at different levels (31, 47) and thus may be implicated in disease pathogenesis. However, despite a strong *HLA-B\*51* association, there was little evidence to suggest that the  $KIR3DL1/S1$  genotype associations found in this study are mediated either by Bw4 or *HLA-B\*51* (Fig. 2). There are two possible explanations for this finding; first, the mechanisms by which the  $KIR3DL1^{LOW}/KIR3DS1$  functional genotype contribute toward disease risk are not directly due to an



**FIGURE 3.** Square markers represent risk genotypes, and circular markers represent protective genotypes. The size of the markers are proportional to the size of the subgroups. The protective  $KIR3DL1^{HIGH}/KIR3DL1^{NULL}$  genotype was associated with a reduced risk of MC disease, whereas the risk  $KIR3DL1^{LOW}/KIR3DS1$  genotype was associated with an increased risk of ophthalmic disease.

inhibitory interaction between  $KIR3DL1$ –Bw4 but rather by indirect mechanisms. Whereas protective effects conferred from the  $KIR3DL1^{HIGH}/KIR3DL1^{NULL}$  functional genotype are mediated via a direct  $KIR3DL1$ –Bw4 interaction. Second, it may be that the  $KIR3DL1$ –Bw4 interaction is integral to both risk and protective effects but that there is a lack of power to detect the risk effects within the *HLA-B\*51*<sup>+</sup> group. It should be noted that the number of *HLA-B\*51*<sup>+</sup> individuals with either the risk or protective functional genotype was small, and this study is unlikely to be sufficiently powered to exclude involvement of *HLA-B\*51* in  $KIR3DL1/S1$  interactions. However, it is possible that  $KIR3DL1/S1$  is affecting disease susceptibility in ways that are independent of Bw4.  $KIR3DL1$ –Bw4 interactions have been extensively investigated and found to be highly variable, depending both on the allotype of  $KIR3DL1$  and on effects outside the Bw4 binding site (50). These interactions are complex and not easily studied in a project designed to analyze the allelic variation of  $KIR3DL1$ .

BD is a heterogeneous condition. Many patients will only ever experience orogenital ulceration, whereas others progress to develop blinding uveitis or inflammatory brain disease. In this study, two low-expressing  $KIR3DL1$  alleles (*\*00501* and *00701*) were both found to confer risk in combination with  $KIR3DS1$ . In the MC group, there was no effect from  $KIR3DL1*005$ , whereas in the ophthalmic group, both alleles contributed to disease susceptibility.  $KIR3DL1*007$  was found less frequently in both groups, but its effect size was between 5 and 10 times greater than  $KIR3DL1*005$ .  $KIR3DL1$  comprises three ancestral lineages:  $KIR3DS1*01301$ ,  $KIR3DL1*005$ , and  $KIR3DL1*015$  (47). From these lineages, selection pressures have led to an abundance of  $KIR3DL1$  allotypes. The  $KIR3DL1*015$  lineage is most diverse in Africans, whereas the  $KIR3DL1*005$  lineage is more common in Caucasians (30). Whereas  $KIR3DL1*005$  has remained stable for more than three million years,  $KIR3DL1*007$  segregated as a clade from  $KIR3DL1*015$  more recently. It is notable in our cohort that  $KIR3DL1*015$  does not associate with BD, whereas  $KIR3DL1*007$  with its narrow binding pattern and  $KIR3DL1*005$  with its broader specificity do. Despite these biological differences, both receptors are expressed at low levels on the cell surface and confer weak inhibition of target cell lysis (51, 52).

Although this is the first study, to our knowledge, to examine  $KIR3DL1/S1$  alleles in BD, our findings correlate with published data on other inflammatory diseases. In 2016, Ahn et al. examined the frequency of  $KIR3DL1$  allotypes in 203 patients with psoriasis and 111 HC of European descent. Their results suggested that the presence of a weakly inhibitory  $KIR3DL1$  allotype increased the risk of developing psoriasis, whereas  $KIR3DL1*004$  was protective (22). Similarly, Díaz-Peña et al. (53) found that the

combination of *KIR3DS1\*013* and *KIR3DL1\*004* protects against ankylosing spondylitis.

Several other factors are involved in HLA-B\*51 activity, including endoplasmic reticulum aminopeptidase-1 (ERAP-1), which cleaves peptides to fit into class I molecules. Polymorphisms in ERAP-1 have been associated with BD, and ERAP-1 variants influence the binding of a low affinity peptidome, which may be relevant in BD (54, 55). Moreover, NK cells require only a few degranulation events (around 10% of the total lytic granules) to kill target cells. Such killing was dependent on the size of granules and the speed at which they are released (56). Activating and inhibitory KIR have been shown to have distinct nanocluster formation on the cell surface controlled by their transmembrane sequence. KIR2DS1 was found to have larger clusters than KIR2DL1 and induce greater downstream signaling (57). Whether KIR allotypes would influence these processes is a possible mechanism to be investigated.

To conclude, this is the first study, to our knowledge, to analyze the effect of KIR3DL1 allotypes in BD. We found *HLA-B\*51* to be associated with BD in a large United Kingdom-based cohort and confirm that *HLA-A\*26* is a risk allele in this population (58) and found *HLA-B\*08* and *A\*25* are also implicated. We also confirm that *HLA-B\*58* and *HLA-A\*33* are protective (38, 39). *KIR3DL1* allele-level analysis revealed the *KIR3DL1<sup>LOW</sup>/KIR3DS1* functional genotype to be implicated in disease pathogenesis and *KIR3DL1<sup>HIGH</sup>/KIR3DL1<sup>NULL</sup>* to be protective.

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## Disclosures

The authors have no financial conflicts of interest.

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