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RESEARCH ARTICLE

Contribution of functional *KIR3DL1* to ankylosing spondylitis

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Increasing evidence points to a role for killer immunoglobulin-like receptors (KIRs) in the development of autoimmune diseases. In particular, a positive association of *KIR3DS1* (activating receptor) and a negative association of *KIR3DL1* (inhibitory receptor) alleles with ankylosing spondylitis (AS) have been reported by several groups. However, none of the studies analyzed these associations in the context of functionality of polymorphic *KIR3DL1*. To better understand how the *KIR3DL1/3DS1* genes determine susceptibility to AS, we analyzed the frequencies of alleles and genotypes encoding functional (*KIR3DL1*F*) and non-functional (*KIR3DL1*004*) receptors. We genotyped 83 AS patients and 107 human leukocyte antigen (HLA)-B27-positive healthy controls from the Russian Caucasian population using a two-stage sequence-specific primer PCR, which distinguishes *KIR3DS1*, *KIR3DL1*F* and *KIR3DL1*004* alleles. For the patients carrying two functional *KIR3DL1* alleles, those alleles were additionally genotyped to identify *KIR3DL1*005* and *KIR3DL1*007* alleles, which are functional but are expressed at low levels. *KIR3DL1* was negatively associated with AS at the expense of *KIR3DL1*F* but not of *KIR3DL1*004*. This finding indicates that the inhibitory *KIR3DL1* receptor protects against the development of AS and is not simply a passive counterpart of the segregating *KIR3DS1* allele encoding the activating receptor. However, analysis of genotype frequencies indicates that the presence of *KIR3DS1* is a more important factor for AS susceptibility than the absence of *KIR3DL1*F*. The activation of either natural killer (NK) or T cells *via* the KIR3DS1 receptor can be one of the critical events in AS development, while the presence of the functional KIR3DL1 receptor has a protective effect. Nevertheless, even individuals with a genotype that carried two inhibitory *KIR3DL1* alleles expressed at high levels could develop AS.
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INTRODUCTION

The etiology of ankylosing spondylitis (AS) is thought to have a significant oligogenic component, with major input coming from the human leukocyte antigen (HLA)-B27.[1–3] Indeed, more than 90% of patients with AS are born with the *HLA-B27* gene, while only 5–15% of carriers are present in the total population.[4,5] Nevertheless, twin studies indicate that *HLA-B27* contributes to only 16% of the total genetic risk,[6] suggesting that AS has multiparameter heritability. Deciphering the heritable factors that contribute to AS is important in understanding the mechanisms of disease initiation and progression.

Several genetic polymorphisms have been reported to be associated with AS, including polymorphisms in the non-MHC genes *IL23R* and *ERAP1*.[7–13] Another group of genes with polymorphisms that likely contribute to AS and other *HLA-B27*-associated diseases is the killer immunoglobulin-like receptor (KIR) genes.[12,14–16] These genes are located on chromosome region 19q13.4, an area that has been implicated in AS development by whole genome scans.[12,14–16]. KIRs are expressed on the surface of natural killer (NK) cells and a subpopulation of T cells.[15,17–20] On NK cells, KIRs play a key role in balancing the activating and inhibitory signals that determine the NK cell response, allowing NK cells to scan for the presence of MHC class I molecules and their antigenic load on target cells.[15, 17–19] The role of KIRs expressed on a minor subpopulation of T cells is less clear. KIR–MHC class I interaction can inhibit cytolytic activity and cytokine production in T cells, but the level of KIR-mediated inhibition of T-cell effector functions depends on the strength of T-cell antigen receptor stimulation.[20–22] Therefore, consistent with the role of KIRs on NK cells, KIRs probably regulate the activation and inhibition of specific T-cell subpopulation(s).

The inheritance of certain HLA and KIR alleles has an important impact on both protection against viral infections and predisposition to autoimmune diseases.[15,18,23,24] A number of studies has been conducted to identify possible associations between autoimmune conditions and KIR genes.[25–35] In particular, a number of works demonstrate

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the role of KIRs in spondyloarthropathies.[16] Among the numerous KIR genes, the *KIR3DL1/3DS1* locus is of particular interest with respect to AS because KIR3DL1 is the only KIR that recognizes HLA-B molecules of the Bw4 serotype, including *HLA-B27*.[36] KIR3DL1 is an inhibitory receptor that interacts with *HLA-Bw4* to suppress the cytolytic capacity of NK or T cells[36–38] and likely plays a role in protecting against autoimmunity.[15,23] A counterpart of KIR3DL1 is the activating receptor, KIR3DS1. *KIR3DL1* and *KIR3DS1* have allelic relationships and are commonly considered alleles of the same KIR gene (IPD-KIR sequence database: http://www.ebi.ac.uk/ipd/kir/). Therefore, possible individual genotypes are *3DL1/3DL1*, *3DL1/3DS1* or *3DS1/3DS1* (rare duplications of *KIR3DL1/3DS1* genes have also been described[39]). In Caucasians, the frequency of *KIR3DL1* is 79% and that of *KIR3DS1* is 21%.[40,41]

Inhibitory KIR3DL1 and activating KIR3DS1 differ by the motifs found in their intracytoplasmic tails, which contain either immunoreceptor tyrosine inhibitory motifs or motifs enabling interaction with activating adaptor proteins, such as DAP12.[42] Conversely, the extracellular domains of KIR3DL1 and KIR3DS1 are strongly homologous. Therefore, although the particular ligand for KIR3DS1 has not yet been identified,[43] KIR3DS1 is thought to interact with HLA-Bw4 carrying a specific load of exogenous peptides. Indeed, the interaction of KIR3DS1 with HLA-Bw4 has been shown to delay the progression of AIDS after HIV-1 infection.[44,45]

Several recent works support the involvement of the *KIR3DL1/3DS1* locus in AS development. The *KIR3DS1* allele occurs more frequently and the *KIR3DL1* allele less frequently in AS patients than in *HLA-B27*-positive controls from Caucasian populations in Spain,[31] Portugal,[31] China[32] and Thailand.[32] Another group also reported that *KIR3DS1* is significantly more frequent in AS patients.[33] In contrast, no increase in *KIR3DS1* carriers among AS patients was noted in a study cohort from the United Kingdom.[34] The contradictory data may be explained by the different inclusion criteria used in each study.

It is important to note that none of the studies analyzed associations of the polymorphic *KIR3DL1* allele in the context of its functionality. Indeed, to perform its inhibitory function, KIR3DL1 must be expressed at the cell surface. At the same time, different *KIR3DL1* alleles are expressed at least at three different levels: low (*KIR3DL1*005*, **006* and **007*), high (*KIR3DL1*001*, **002*, **003*, **008*, **009*, **015* and **020*), and not expressed/null (*KIR3DL1*004*).[46–49] The latter allele, *KIR3DL1*004*, carries the amino acid polymorphism Ser86Leu, which interrupts proper protein folding and leads to its intracellular retention.[50] Because KIR3DL1*004 is absent from the cell surface, it cannot provide inhibitory signaling through recognition of an HLA-Bw4, such as HLA-B27. Thus, *KIR3DL1*004* indirectly supports a more potent activation of immune cells. In agreement with this, a slower progression of AIDS has been noted in individuals with the *HLA-Bw4/KIR3DL1*004* genotype.[51] The functionality of *KIR3DL1* may also be connected to the etiology of AS and other *HLA-B27*-associated diseases. In Caucasians, *KIR3DL1*004* is rather frequent, comprising 16–18% of *KIR3DL1/3DS1* alleles. Approximately 30% of Caucasians carry *KIR3DL1*004* and thus its contribution to *KIR3DL1* associations may be significant.[40,41]

Here, we investigated whether functional *KIR3DL1* alleles, the nonfunctional allele *KIR3DL1*004* and the activating allele *KIR3DS1* were positively or negatively associated with AS in a Russian Caucasian cohort. Clarifying *KIR3DL1/3DS1* associations by accounting for the functional activity of *KIR3DL1* should provide a better understanding of the events occurring during activation of NK or T cells that may be critical to the initiation of AS.

MATERIALS AND METHODS

Patients and controls

AS patients were diagnosed at the Institute of Rheumatology, RAMS (Russian Academy of Medical Sciences), Moscow, Russia, in accordance with modified New York criteria (1984).[52] Radiographs of the pelvis and lumbar spine were obtained from all patients. Inclusion criteria were as follows: all patients had a bilateral sacroiliitis of grade II or higher, and only *HLA-B27*-positive patients were selected for the study. The venous blood samples from the *HLA-B27*-positive controls were collected from a group of healthy, unrelated individuals living in the Moscow area. Venous blood was collected from adult patients (aged 21–63 years) without chronic disease during medical and/or diagnostic procedures. The presence of the *HLA-B27* allele in controls and AS patients was confirmed by sequence specific primer PCR (SSP-PCR) and flow cytometry. Both patients and controls gave written informed consent prior to enrolling in the study. The study was conducted according to the Declaration of Helsinki.

Genotyping

Peripheral blood mononuclear cells were isolated from fresh whole peripheral blood samples using Ficoll–Urografin density gradient separation. Genomic DNA was prepared from peripheral blood mononuclear cells using the DNA extraction kit, Diatom DNA Prep (Isogen, Moscow, Russia). For genotyping, *KIR3DL1* and *KIR3DS1* were considered alleles of the same KIR gene. *KIR3DL1*/*KIR3DS1* typing of DNA samples was performed using SSP-PCR as described previously.[53] Results were confirmed by independent SSP-PCR using two pairs of primers specific for *KIR3DL1* (KIR3DL1_DS1_For2 and KIR3DL1_Rev3) or *KIR3DS1* (KIR3DL1_DS1_For2 and KIR3DS1_Rev3). To discriminate between *KIR3DL1* allelic variants carrying Leu86 or Ser86, we used the KIR3DL1_DS1_For2–KIR3DL1_Rev3 PCR amplification product as a template for the two nested SSP-PCR reactions using primer pairs specific for *KIR3DL1-Leu86* (KIR3DL1_For3 and KIR3DL1_Leu86Rev) or *KIR3DL1-Ser86* (KIR3DL1_For3 and KIR3DL1_Ser86Rev). The same nested SSP-PCR approach was employed to identify the allelic variants *KIR3DL1*005* and *KIR3DL1*007* (see Supplementary Table 1 for primer sequences).

Quality guarantee measures

Genotyping results were reproduced at least twice for each of the individual genomes using one or both variations of the SSP-PCR assay. In addition, a subset of three healthy and three AS-affected individual genomes was analyzed by comprehensive accurate sequencing of *KIR3D* regions flanked by KIR3DL1_DS1_For2 and KIR3D_Rev2 (see Supplementary Table 1 for primer sequences). As a result, several variants of the *KIR3DL1* allele, including *KIR3DL1*004* and *KIR3DS1* alleles, were identified. The sequencing results perfectly confirmed the genotyping by SSP-PCR for each of the analyzed individuals.

Statistical analysis

Allelic and genotypic frequencies were calculated by direct counting. Hardy–Weinberg equilibrium for *KIR3DL1* and *KIR3DS1* alleles was tested using Genome Data Analysis software. Associations were analyzed using the χ^2 test with Yate's correction or Fisher's exact test. Values of $P<0.05$ were considered statistically significant. The odds ratio (OR) was calculated by the cross-product ratio, and the corresponding 95% confidence interval was determined.

Table 1 Allelic frequencies of *KIR3DL1* and *KIR3DS1* in Caucasian AS patients and healthy controls from Russian, Azorean, and Spanish populations[a]

	Frequency in AS patients % (number of alleles)			*Frequency in control individuals % (number of alleles)*			*P value Russian AS versus Russian controls*	*OR (95% CI) Russian AS versus Russian controls*	*P value Combined Caucasian cohorts[b]*	*OR (95% CI) Combined Caucasian cohorts[b]*
Allele	Russian (2*n*=166)	Spanish (2*n*=142)	Azorean (2*n*=110)	Russian (2*n*=214)	Spanish (2*n*=210)	Azorean (2*n*=114)				
3DL1	69.3% (115)	57% (81)	64.5% (71)	81.3% (174)	77.6% (163)	82.4% (94)	<0.01	0.52 (0.32–0.84)	$<1\times10^{-7}$	0.44 (0.33–0.60)
3DS1	30.7% (51)	42.9% (61)	35.4% (39)	18.7% (40)	22.3% (47)	17.5% (20)	<0.01	1. 93 (1.20–3.11)	$<1\times10^{-7}$	2.28 (1.70–3.05)

[a] Data for the Azorean and Spanish populations taken from Ref. 31.
[b] Calculated for the combined cohorts of Russian, Spanish and Azorean AS patients and controls.
Abbreviations: AS, ankylosing spondylitis; CI, confidence interval; KIR, killer immunoglobulin-like receptor; OR, odds ratio.

RESULTS

Contribution of the *KIR3DL1/3DS1* gene to AS development

To assess the possible association of the *KIR3DL1* polymorphism with AS, we typed 83 *HLA-B27*-positive Russian Caucasian patients with AS and 107 *HLA-B27*-positive healthy controls.

As a first step, SSP-PCR analysis was used to distinguish *KIR3DL1* and *KIR3DS1* alleles, as has been performed in previous work.[31–34] *KIR3DL1* and *KIR3DS1* allele distribution was analyzed in both cohorts and showed no significant deviation from Hardy–Weinberg equilibrium ($P>0.05$). We found that the *KIR3DL1* allele was underrepresented ($P<0.01$, OR=0.52) and the *KIR3DS1* allele was overrepresented ($P<0.01$, OR=1.93) in the AS group compared to healthy controls (Table 1). Therefore, our data confirm that AS is associated with an increased frequency of *KIR3DS1* and a decreased frequency of *KIR3DL1*, as has been reported for other study populations.[31–33]

We also confirmed that the frequency of the *KIR3DL1/KIR3DL1* genotype was decreased ($P=0.005$, OR=0.42) and the frequency of the *KIR3DL1/KIR3DS1* genotype was increased ($P=0.01$, OR=2.27) in AS patients compared to *HLA-B27*-positive controls. Analysis of combined Russian, Spanish and Azorean Caucasian populations also showed a statistically significant increase in the *KIR3DS1/KIR3DS1* genotype frequency among AS patients ($P=0.01$, OR=2.35; Table 2). Thus, the findings in our study and previous work support an important role for the *KIR3DL1/3DS1* gene in the etiology of AS.

However, it remained unclear which allele provided the most prominent input. Is the predisposition to AS attributable to the absence of *KIR3DL1* (which protects against autoimmunity by interacting with *HLA-B27*) or the presence of *KIR3DS1* (which promotes stimulation of NK and/or T cells), or are both conditions required? The genotyping studies conducted above could not answer this question, as *KIR3DL1* and *KIR3DS1* are segregating alleles.

Protective role of the functional *KIR3DL1* allele

Although most KIR genes seem to be expressed stochastically and independently of one another, their expression is tightly regulated.[54] In particular, it has been demonstrated that in *KIR3DS1/KIR3DL1* heterozygous donors, most NK cells express either *KIR3DS1* only or *KIR3DL1* only.[55] Selective expression of either inhibitory or activating KIRs has also been recently reported for a minor subset of $CD4^+$ T cells.[56] Therefore, the association of the *KIR3DS1* allele with AS could be explained by a statistically decreased frequency of NK or T cells expressing *KIR3DL1* in *KIR3DS1*-positive individuals, rather than by a direct functional activation of *KIR3DS1*-expressing immune cells, especially as KIR3DS1 has not yet been shown to interact with HLA-Bw4.[43] To investigate this possibility, we performed second-stage analysis using nested SSP-PCR, which allowed us to distinguish *KIR3DL1* alleles known to be functionally expressed on the cell surface (including *KIR3DL1*001–003*, **005–009*, **015* and **020*; designated *KIR3DL1*F*) from the non-functional allele *KIR3DL1*004*. The aggregate frequency of rare *KIR3DL1* alleles other than *KIR3DL1*F* and **004* is below 1% and therefore their input was ignored.

Because the *KIR3DL1*004* receptor plays neither a stimulatory nor an inhibitory role, we expected that its allelic frequency would depend on the pressure of the functional alleles that play a dominant role. Indeed, if expression of activating *KIR3DS1* plays a dominant role in disease development (direct association), then its allelic frequency should be increased at the expense of both *KIR3DL1*F* and *KIR3DL1*004* independently of the functionality of these alleles. Alternatively, if the absence of the protective *KIR3DL1*F* allele plays

Table 2 Genotypic frequencies of *KIR3DL1* and *KIR3DS1* in Caucasian AS patients and healthy controls in Russian, Azorean and Spanish populations[a]

	Frequency in AS patients, % (no. of patients)			*Frequency in control individuals, % (no. of individuals)*			*P value Russian AS versus Russian controls*	*OR (95% CI) Russian AS versus Russian controls*	*P value Combined Caucasian cohorts[b]*	*OR (95% CI) Combined Caucasian cohorts[b]*
Genotype	Russian (*n*=83)	Spanish (*n*=71)	Azorean (*n*=55)	Russian (*n*=107)	Spanish (*n*=105)	Azorean (*n*=57)				
3DL1/3DL1	48.2% (40)	35.2% (25)	36.3% (20)	69.2% (74)	61.9% (65)	68.4% (39)	0.005	0.42 (0.23–0.75)	$<1\times10^{-7}$	0.35 (0.24–0.51)
3DL1/3DS1	42.2% (35)	43.6% (31)	56.1% (31)	24.3% (26)	31.4% (33)	28% (16)	0.01	2.27 (1.22–4.23)	$<1\times10^{-4}$	2.24 (1.53–3.28)
3DS1/3DS1	9.6% (8)	21.1% (15)	7.2% (4)	6.5% (7)	6.6% (7)	3.5% (2)	NS	—	0.01	2.35 (1.23–4.48)

[a] Data for the Azorean and Spanish populations taken from Ref. 31.
[b] Calculated for the combined cohorts of Russian, Spanish and Azorean AS patients and controls.
Abbreviations: AS, ankylosing spondylitis; CI, confidence interval; KIR, killer immunoglobulin-like receptor; NS, not significant; OR, odds ratio.

Table 3 Allelic frequencies of *KIR3DL1*F*, *KIR3DL1*004* and *KIR3DS1* in AS patients and healthy controls in Russian Caucasian population

	Frequency, % (no. of alleles)			
Allele	AS (2*n*=166)	Control (2*n*=214)	*P value*	*OR (95% CI)*
*3DL1*F*	48.2% (80)	63.1% (135)	0.005	0.54 (0.36–0.82)
*3DL1*004*	21.1% (35)	18.2% (39)	NS	—
3DS1	30.7% (51)	18.7% (40)	<0.01	1.94 (1.21–3.12)

Abbreviations: AS, ankylosing spondylitis; CI, confidence interval; KIR, killer immunoglobulin-like receptor; NS, not significant; OR, odds ratio.

a dominant role, then the frequency of this allele should be decreased in AS patients in favor of both *KIR3DS1* and *KIR3DL1*004* independently of their ability to transfer an activating signal (*KIR3DS1*) or inability to transfer any signal (*KIR3DL1*004*).

Remarkably, results from the analysis of the allelic frequencies of *KIR3DL1*F*, *KIR3DL1*004* and *KIR3DS1* in AS patients supported neither of the two hypotheses. As expected, AS patients had a lower frequency of *KIR3DL1*F* (P=0.005, OR=0.54) and a higher frequency of *KIR3DS1* (P<0.01, OR=1.94) alleles compared to healthy controls. However, the frequency of the *3DL1*004* allele between AS patients and healthy controls was approximately equal (Table 3). The frequency of *KIR3DL1*004* carriers was also equal between patients with AS and healthy controls (Table 4).

Thus, neither allelic nor carrier frequency of *KIR3DL1*004* correlated with frequency of the *KIR3DL1*F* or *KIR3DS1* allele. Both allelic and carrier frequency of *KIR3DL1*004* demonstrated remarkable similarity between patients and controls. This result indicates that the input of the activating *KIR3DS1* allele is not the only factor in AS initiation, and that the inhibitory *KIR3DL1*F* allele plays an antagonistic, protective role in disease development.

DISCUSSION

Typing of *KIR3DL1*F/3DL1*004/3DS1* allowed us to distinguish among six functionally distinct genotypes: *3DL1*F/3DL1*F*, *3DL1*F/3DL1*004*, *3DL1*004/3DL1*004*, *3DL1*F/3DS1*, *3DL1*004/3DS1* and *3DS1/3DS1* (Table 5). Although a statistically reliable interpretation of distribution of these genotypes requires a much higher number of patients in the study, the analysis still allows for preliminary conclusions. Among these genotypes, *3DL1*F/3DL1*F* was the only genotype assuring that each immune cell expressing the *KIR3DL1/3DS1* locus would present the inhibitory KIR3DL1 receptor at its surface. In the healthy population, this genotype had the highest frequency, constituting approximately 37–40% of individuals (Ref. 41 and Table 5). Both genotypes *3DL1*F/3DL1*004* and *3DL1*F/3DS1* accounted for immune cells that may have no functional inhibitory KIR3DL1 receptor on their surface despite upregulated expression of the gene. Finally, immune cells of the individuals with the *3DL1*004/3DL1*004*, *3DL1*004/3DS1* or *3DS1/3DS1* genotypes would not express the KIR3DL1 receptor at their surface.

Table 4 Frequency of *KIR3DL1*F*, *KIR3DL1*004* and *KIR3DS1* carriers in AS patients and healthy controls in the Russian Caucasian population

	Frequency, % (no. of individuals)			
Alleles	AS (*n*=83)	Control (*n*=107)	*P value*	*OR (95% CI)*
*3DL1*F*	75.9% (63)	83.2% (89)	NS	—
*3DL1*004*	36.1% (30)	31.8% (34)	NS	—
3DS1	51.8% (43)	30.8% (33)	0.03	1.72 (1.08–2.92)

Abbreviations: AS, ankylosing spondylitis; CI, confidence interval; KIR, killer immunoglobulin-like receptor; NS, not significant; OR, odds ratio.

Table 5 Genotypic frequencies of *KIR3DL1*F*, *KIR3DL1*004* and *KIR3DS1* in AS patients and healthy controls in the Russian Caucasian population

	Frequency, % (no. of individuals)			
Genotype	AS (*n*=83)	Control (*n*=107)	*P value*	*OR (95% CI)*
*3DL1*F/3DL1*F*	20.5% (17)	43.0% (46)	<0.002	0.34 (0.18–0.66)
*3DL1*F/3DL1*004*	21.7% (18)	21.5% (23)	NS	—
*3DL1*004/3DL1*004*	6.0% (5)	4.7% (5)	NS	—
*3DL1*F/3DS1*	33.7% (28)	18.7% (20)	0.03	2.22 (1.14–4.31)
*3DL1*004/3DS1*	8.4% (7)	5.6% (6)	NS	—
3DS1/3DS1	9.6% (8)	6.5% (7)	NS	—

Abbreviations: AS, ankylosing spondylitis; CI, confidence interval; KIR, killer immunoglobulin-like receptor; NS, not significant; OR, odds ratio.

Hypothesizing that the protective role of the KIR3DL1*F receptor drives the association of the locus with AS, we would expect to see the following changes in genotypic frequencies in AS patients:

1. a decrease in the percentage of *3DL1*F/3DL*F* genotypes;
2. a decrease, albeit to a lesser extent, in the percentage of *3DL1*F/3DL1*004* and *3DL1*F/3DS1* genotypes;
3. a uniform increase in the percentage of *3DL1*004/3DL1*004*, *3DL1*004/3DS1* and *3DS1/3DS1* genotypes.

Conversely, hypothesizing that the activating KIR3DS1 receptor drives the association of the locus with AS, we would expect the following alterations in frequencies in AS patients:

1. an increase in the percentage of *3DS1/3DS1* genotypes;
2. a smaller increase in the percentage of *3DL1*004/3DS1* and *3DL1*F/3DS1* genotypes;
3. a uniform decrease in the percentage of *3DL1*F/3DL*F*, *3DL1*F/3DL1*004* and *3DL1*004/3DL1*004* genotypes.

Our data generally support the latter hypothesis. Indeed, the frequency of the *3DL1*F/3DS1* genotype was greater in AS patients than in healthy controls (P=0.03, OR=2.22; Table 5), arguing against the first hypothesis. In general, the frequency of genotypes carrying the *KIR3DS1* allele was increased in AS patients (P=0.03, OR=1.72; Table 4) independently of the presence or absence of the functional inhibitory *KIR3DL1*F* allele (Table 5). Therefore, we conclude that the input of the activating *KIR3DS1* allele is probably the strongest in the hypothetical checkpoint that precedes initiation of AS.

At the same time, the functional role of the KIR3DL1 receptor demonstrated by analysis of the allelic and carrier frequencies (Tables 3 and 4) was also notable and presumably affects genotype frequencies. Indeed, in AS patients, the decrease in the frequency of the 'always functional' genotype, *3DL1*F/3DL1*F*, was prominent and statistically significant (P<0.002, OR=0.34), while the frequency of the *3DL1*F/3DL1*004* genotype was approximately equal between patients and controls (Table 5).

In the context of the proposed protective role of the functional KIR3DL1 receptor, a plausible explanation for disease development in *3DL1*F/3DL1*F* AS patients could be the presence of either the

*KIR3DL1*005* or the *KIR3DL1*007* allele, which are known to be expressed at low levels.[46–49] Another allele expressed at low levels, *KIR3DL1*006*, is not characteristic of the Caucasian population.[40,41] To verify if the low expression level alleles are present in *3DL1*F/3DL1*F* AS patients, we additionally typed them for the presence of *KIR3DL1*005* and *KIR3DL1*007* alleles. Notably, the typing revealed that 9 of 17 *3DL1*F/3DL1*F* patients carried low expression alleles (5× *3DL1*F/3DL1*005*, 3× *3DL1*005/3DL1*005* and 1× *3DL1*F/3DL1*007*). However, the remaining eight patients had two functional *KIR3DL1* alleles, both expressed at a high level. Therefore, even those *3DL1*F/3DL1*F* genotypes that carried both *KIR3DL1* alleles expressed at a high level still did not guarantee protection against AS.

In summary, analysis of *KIR3DL1*F*, *KIR3DL1*004* and *KIR3DS1* allelic and genotypic frequencies in AS patients showed that both inhibitory KIR3DL1*F receptors and the activating receptor, KIR3DS1, functionally contribute to the probability of disease development. Nevertheless, input of the KIR3DS1 receptor is probably more important, as it followed from the analysis of genotypic frequencies. The protective function of *KIR3DL1*F* likely becomes realized when KIR3DS1 is absent from the surface of the immune cell. Therefore, the interaction between KIR3DS1 and HLA-B27 is probably a key event that should be investigated to uncover the etiology of AS and other *HLA-B27*-associated diseases. Our findings should be confirmed by the analysis of an expanded cohort of AS patients, which should provide a more profound understanding of the role of KIR receptors in autoimmunity. In general, the analysis of linked alterations in frequencies of functional and non-functional alleles should provide a deeper understanding of how the balance of activating and inhibitory receptors functionally determines susceptibility to autoimmune diseases.

Note: Supplementary information is available on the Cellular & Molecular Immunology website (http://www.nature.com/cmi/).

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1 Caffrey M, Brewerton DA, Hart FD, James DC. Human lymphocyte antigens as a possible diagnostic aid in ankylosing spondylitis. *J Clin Pathol* 1973; **26**: 387.
2 Brewerton DA, Hart FD, Nicholls A, Caffrey M, James DC, Sturrock RD. Ankylosing spondylitis and HL-A 27. *Lancet* 1973; **1**: 904–907.
3 Schlosstein L, Terasaki PI, Bluestone R, Pearson CM. High association of an HL-A antigen, W27, with ankylosing spondylitis. *N Engl J Med* 1973; **288**: 704–706.
4 Smith JA, Marker-Hermann E, Colbert RA. Pathogenesis of ankylosing spondylitis: current concepts. *Best Pract Res Clin Rheumatol* 2006; **20**: 571–591.
5 Bowness P. HLA B27 in health and disease: a double-edged sword? *Rheumatology (Oxford)* 2002; **41**: 857–868.
6 Sims AM, Wordsworth BP, Brown MA. Genetic susceptibility to ankylosing spondylitis. *Curr Mol Med* 2004; **4**: 13–20.
7 Burton PR, Clayton DG, Cardon LR, Craddock N, Deloukas P, Duncanson A *et al*. Association scan of 14,500 nonsynonymous SNPs in four diseases identifies autoimmunity variants. *Nat Genet* 2007; **39**: 1329–1337.
8 Brown MA. Breakthroughs in genetic studies of ankylosing spondylitis. *Rheumatology (Oxford)* 2008; **47**: 132–137.
9 Harvey D, Pointon JJ, Evans DM, Karaderi T, Farrar C, Appleton LH *et al*. Investigating the genetic association between ERAP1 and ankylosing spondylitis. *Hum Mol Genet* 2009; **18**: 4204–4212.
10 Tsui FW, Haroon N, Reveille JD, Rahman P, Chiu B, Tsui HW *et al*. Association of an ERAP1 ERAP2 haplotype with familial ankylosing spondylitis. *Ann Rheum Dis* 2010; **69**: 733–736.
11 Maksymowych WP, Inman RD, Gladman DD, Reeve JP, Pope A, Rahman P. Association of a specific ERAP1/ARTS1 haplotype with disease susceptibility in ankylosing spondylitis. *Arthritis Rheum* 2009; **60**: 1317–1323.
12 Brown MA. Genetics and the pathogenesis of ankylosing spondylitis. *Curr Opin Rheumatol* 2009; **21**: 318–323.
13 Reveille JD. The genetic basis of ankylosing spondylitis. *Curr Opin Rheumatol* 2006; **18**: 332–341.
14 Carter KW, Pluzhnikov A, Timms AE, Miceli-Richard C, Bourgain C, Wordsworth BP *et al*. Combined analysis of three whole genome linkage scans for Ankylosing Spondylitis. *Rheumatology (Oxford)* 2007; **46**: 763–771.
15 Kulkarni S, Martin MP, Carrington M. The Yin and Yang of HLA and KIR in human disease. *Semin Immunol* 2008; **20**: 343–352.
16 Diaz-Pena R, Blanco-Gelaz MA, Lopez-Larrea C. KIR genes and their role in spondyloarthropathies. *Adv Exp Med Biol* 2009; **649**: 286–299.
17 Gardiner CM. Killer cell immunoglobulin-like receptors on NK cells: the how, where and why. *Int J Immunogenet* 2008; **35**: 1–8.
18 Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol* 2002; **20**: 217–251.
19 Parham P. Immunogenetics of killer cell immunoglobulin-like receptors. *Mol Immunol* 2005; **42**: 459–462.
20 Anfossi N, Doisne JM, Peyrat MA, Ugolini S, Bonnaud O, Bossy D *et al*. Coordinated expression of Ig-like inhibitory MHC class I receptors and acquisition of cytotoxic function in human $CD8^+$ T cells. *J Immunol* 2004; **173**: 7223–7229.
21 de Maria A, Ferraris A, Guastella M, Pilia S, Cantoni C, Polero L *et al*. Expression of HLA class I-specific inhibitory natural killer cell receptors in HIV-specific cytolytic T lymphocytes: impairment of specific cytolytic functions. *Proc Natl Acad Sci USA* 1997; **94**: 10285–10288.
22 van der Veken LT, Campelo MD, van der Hoorn MA, Hagedoorn RS, van Egmond HM, van Bergen J *et al*. Functional analysis of killer Ig-like receptor-expressing cytomegalovirus-specific $CD8^+$ T cells. *J Immunol* 2009; **182**: 92–101.
23 Parham P. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol* 2005; **5**: 201–214.
24 Sambrook JG, Bashirova A, Palmer S, Sims S, Trowsdale J, Abi-Rached L *et al*. Single haplotype analysis demonstrates rapid evolution of the killer immunoglobulin-like receptor (KIR) loci in primates. *Genome Res* 2005; **15**: 25–35.
25 Chan AT, Kollnberger SD, Wedderburn LR, Bowness P. Expansion and enhanced survival of natural killer cells expressing the killer immunoglobulin-like receptor *KIR3DL2* in spondylarthritis. *Arthritis Rheum* 2005; **52**: 3586–3595.
26 Nelson GW, Martin MP, Gladman D, Wade J, Trowsdale J, Carrington M. Cutting edge: heterozygote advantage in autoimmune disease: hierarchy of protection/susceptibility conferred by HLA and killer Ig-like receptor combinations in psoriatic arthritis. *J Immunol* 2004; **173**: 4273–4276.
27 Martin MP, Carrington M. KIR locus polymorphisms: genotyping and disease association analysis. *Methods Mol Biol* 2008; **415**: 49–64.
28 Ploski R, Luszczek W, Kusnierczyk P, Nockowski P, Cislo M, Krajewski P *et al*. A role for KIR gene variants other than KIR2DS1 in conferring susceptibility to psoriasis. *Hum Immunol* 2006; **67**: 521–526.
29 Holm SJ, Sakuraba K, Mallbris L, Wolk K, Stahle M, Sanchez FO. Distinct HLA-C/KIR genotype profile associates with guttate psoriasis. *J Invest Dermatol* 2005; **125**: 721–730.
30 Lorentzen AR, Karlsen TH, Olsson M, Smestad C, Mero IL, Woldseth B *et al*. Killer immunoglobulin-like receptor ligand *HLA-Bw4* protects against multiple sclerosis. *Ann Neurol* 2009; **65**: 658–666.
31 Lopez-Larrea C, Blanco-Gelaz MA, Torre-Alonso JC, Bruges Armas J, Suarez-Alvarez B, Pruneda L *et al*. Contribution of *KIR3DL1/3DS1* to ankylosing spondylitis in human leukocyte antigen-B27 Caucasian populations. *Arthritis Res Ther* 2006; **8**: R101.
32 Diaz-Pena R, Blanco-Gelaz MA, Suarez-Alvarez B, Martinez-Borra J, Lopez-Vazquez A, Alonso-Arias R *et al*. Activating KIR genes are associated with ankylosing spondylitis in Asian populations. *Hum Immunol* 2008; **69**: 437–442.
33 Jiao YL, Ma CY, Wang LC, Cui B, Zhang J, You L *et al*. Polymorphisms of KIRs gene and *HLA-C* alleles in patients with ankylosing spondylitis: possible association with susceptibility to the disease. *J Clin Immunol* 2008; **28**: 343–349.
34 Harvey D, Pointon JJ, Sleator C, Meenagh A, Farrar C, Sun JY *et al*. Analysis of killer immunoglobulin-like receptor genes in ankylosing spondylitis. *Ann Rheum Dis* 2009; **68**: 595–598.
35 Zhang BC, Liu Y, Jiao YL, Zhao YR, Li JF. Genotype and haplotype analysis of killer cell immunoglobulin-like receptors in ankylosing spondylitis. *Zhonghua Yi Xue Za Zhi* 2009; **89**: 91–95. Chinese.
36 Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P. The Bw4 public epitope of *HLA-B* molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med* 1995; **181**: 1133–1144.
37 Litwin V, Gumperz J, Parham P, Phillips JH, Lanier LL. NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. *J Exp Med* 1994; **180**: 537–543.
38 Vitale M, Sivori S, Pende D, Augugliaro R, Di Donato C, Amoroso A *et al*. Physical and functional independency of p70 and p58 natural killer (NK) cell receptors for HLA class I: their role in the definition of different groups of alloreactive NK cell clones. *Proc Natl Acad Sci USA* 1996; **93**: 1453–1457.
39 Gomez-Lozano N, Estefania E, Williams F, Halfpenny I, Middleton D, Solis R *et al*. The silent *KIR3DP1* gene (CD158c) is transcribed and might encode a secreted receptor in a minority of humans, in whom the *KIR3DP1*, *KIR2DL4* and *KIR3DL1/KIR3DS1* genes are duplicated. *Eur J Immunol* 2005; **35**: 16–24.
40 Carrington M, Gao X, Norman P. KIR gene allele frequencies in populations from USA (African American) USA (European) and England. *Human Immunology* 2004; **65**: 1191.
41 Available from: www.allelefrequencies.net

42 Lanier LL, Corliss BC, Wu J, Leong C, Phillips JH. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature* 1998; **391**: 703–707.
43 Gillespie GM, Bashirova A, Dong T, McVicar DW, Rowland-Jones SL, Carrington M. Lack of *KIR3DS1* binding to MHC class I Bw4 tetramers in complex with $CD8^+$ T cell epitopes. *AIDS Res Hum Retroviruses* 2007; **23**: 451–455.
44 Martin MP, Gao X, Lee JH, Nelson GW, Detels R, Goedert JJ *et al*. Epistatic interaction between *KIR3DS1* and *HLA-B* delays the progression to AIDS. *Nat Genet* 2002; **31**: 429–434.
45 Alter G, Martin MP, Teigen N, Carr WH, Suscovich TJ, Schneidewind A *et al*. Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. *J Exp Med* 2007; **204**: 3027–3036.
46 Li H, Pascal V, Martin MP, Carrington M, Anderson SK. Genetic control of variegated KIR gene expression: polymorphisms of the bi-directional *KIR3DL1* promoter are associated with distinct frequencies of gene expression. *PLoS Genet* 2008; **4**: e1000254.
47 Gardiner CM, Guethlein LA, Shilling HG, Pando M, Carr WH, Rajalingam R *et al*. Different NK cell surface phenotypes defined by the DX9 antibody are due to *KIR3DL1* gene polymorphism. *J Immunol* 2001; **166**: 2992–3001.
48 Yawata M, Yawata N, Draghi M, Little AM, Partheniou F, Parham P. Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection and modulation of effector function. *J Exp Med* 2006; **203**: 633–645.
49 Pascal V, Yamada E, Martin MP, Alter G, Altfeld M, Metcalf JA *et al*. Detection of KIR3DS1 on the cell surface of peripheral blood NK cells facilitates identification of a novel null allele and assessment of *KIR3DS1* expression during HIV-1 infection. *J Immunol* 2007; **179**: 1625–1633.
50 Pando MJ, Gardiner CM, Gleimer M, McQueen KL, Parham P. The protein made from a common allele of *KIR3DL1* (*3DL1*004*) is poorly expressed at cell surfaces due to substitution at positions 86 in Ig domain 0 and 182 in Ig domain 1. *J Immunol* 2003; **171**: 6640–6649.
51 Martin MP, Qi Y, Gao X, Yamada E, Martin JN, Pereyra F *et al*. Innate partnership of *HLA-B* and *KIR3DL1* subtypes against HIV-1. *Nat Genet* 2007; **39**: 733–740.
52 van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. *Arthritis Rheum* 1984; **27**: 361–368.
53 Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B *et al*. Human diversity in killer cell inhibitory receptor genes. *Immunity* 1997; **7**: 753–763.
54 van Bergen J, Stewart CA, van den Elsen PJ, Trowsdale J. Structural and functional differences between the promoters of independently expressed killer cell Ig-like receptors. *Eur J Immunol* 2005; **35**: 2191–2199.
55 Trundley A, Frebel H, Jones D, Chang C, Trowsdale J. Allelic expression patterns of *KIR3DS1* and *3DL1* using the Z27 and DX9 antibodies. *Eur J Immunol* 2007; **37**: 780–787.
56 Remtoula N, Bensussan A, Marie-Cardine A. Cutting edge: selective expression of inhibitory or activating killer cell Ig-like receptors in circulating $CD4^+$ T lymphocytes. *J Immunol* 2008; **180**: 2767–2771.