

Excess of activating killer cell immunoglobulin-like receptors and lack of HLA-Bw4 ligands: A two-edged weapon in chronic fatigue syndrome

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Abstract. Chronic fatigue syndrome (CFS) is an inflammatory disease of unknown aetiology. Researchers have proposed infectious, neurological and immunological causes of this syndrome. Recently, the xenotropic murine leukemia virus-related virus was detected in 67% of patients with CFS in a US study. This observation is in agreement with one ascertained aspect of the disease: a decreased efficiency in NK cell lytic activity in CFS patients. Here, we analyzed the genomic polymorphism of killer cell immunoglobulin-like receptors (KIRs) and their HLA class I cognate ligands in patients with certified CFS. An excess of KIR3DS1 was found in CFS patients with respect to controls, as well as an increased frequency of the genotype missing KIR2DS5. Forty-four CFS patients and 50 controls also underwent genomic typing for the HLA-ligands. In the patients, a great proportion of KIR3DL1 and KIR3DS1 receptors were found to be missing their HLA-Bw4^{Ile80} binding motif. We hypothesize that an excess of KIR3DS1, combined with an excess of ligand-free KIR3DL1 and KIR3DS1 receptors, may hamper the clearance of a pathogen via NK cells, thus favouring the chronicity of the infection.

Introduction

Chronic fatigue syndrome (CFS) is a debilitating disease characterized by chronic post-exertional fatigue of at least

a 6-month duration that is not resolved with rest, and by the preclusion of other known diseases which cause its symptoms. The most widely used classification criteria are those established by Fukuda in 1994, although efforts have been made to update these (1-4). The diagnosis of CFS is based on a constellation of symptoms and clinical indicators, as no pathognomonic tests are available. The epidemiology of CFS has been investigated and has been found to include a higher prevalence in women than in men and among individuals between the ages of 18-50 years, with little distinction between socio-economic classes, and a prevalence of 0.5% in the US (5-7).

Differences in the onset of symptoms (acute or gradual; after a viral infection such as influenza or mononucleosis or without any prodromal episode), the course of the disease (cyclic, steady or slowly worsening, or with gradual improvement until normal health is restored) and the frequent psychiatric and allergic comorbidity suggest that CFS has different aetiologies. Researchers have proposed infectious, neurological or immunological causes for the syndrome.

A number of viruses, such as enterovirus, Epstein Barr virus and herpesvirus, have been implicated as possible causes of CFS, suggesting an underlying immune deficiency (8). In a recent study, the retrovirus xenotropic murine leukemia virus-related virus (XMRV) was detected by American researchers in the blood cells of patients with CFS (9). This finding was not confirmed in another set of patients originating from the UK (10,11). Population genetic disparities and different pathogenic repertoire may explain why XMRV was not detected in the DNA from UK patients. Preliminary data show that in our Italian population, 39% of patients and 4.9% of controls present the retrovirus (unpublished data). The debate is still ongoing, and several research groups are evaluating the possible role of viruses in CFS (12-14). However, a retroviral infection is a particularly attractive possibility, suggesting an involvement of the host's genetic profile in disease evolution.

A review of the literature has excluded any macroscopic abnormalities in immune cell counts, but there is controversy regarding other aspects of immune system function (15). Our data revealed a pro-inflammatory genotype in patients

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with CFS, with a highly significant increase in TNF- α high producers and a significant decrease in IFN- γ low producers compared to a population of healthy controls (16). Therefore, in a recent study (17), we evaluated the presence of autoantibodies in order to elucidate the underlying genetic and immunologic factors contributing to the pathogenesis of CFS. A high prevalence of anti-endothelial and anti-neuronal cell antibodies was observed. By contrast, anti-NMDA antibodies were not detected in any of the patients. Moreover, we studied the HLA class II gene DRB1 and two polymorphisms of the RAGE gene promoter (18) in association with CFS, and found notable RAGE-HLA DRB1 haplotypes in patients with this syndrome, suggesting an involvement of the MHC region.

Researchers have additionally found two other factors altered in the immune system of CFS patients: increased expression of CD28⁺ T lymphocytes and decreased NK cell function (19). NK cells play an essential role at the interface between innate and adaptive immunity. Under normal conditions, through the inhibitory subset of killer immunoglobulin-like receptors (KIRs) expressed on their surface, NK cells recognize self MHC class I molecules (KIR-ligands) on the cell surface, and send an inhibitory signal to avoid the elimination of normal cells. However, when KIRs encounter a damaged cell with missing or underexpressed MHC class I molecules, NK cells are not inhibited and proceed to kill the damaged cell. This concept is known as the missing-self hypothesis (20). The performance of NK cells is guaranteed by properly functioning HLA-KIR interaction, and we suspect a dysregulation at this level in CFS. Therefore, we analysed variability in the presence/absence of KIRs genes and HLA class I ligands in an Italian population to investigate this polymorphism, which is a possible cause of NK cell lytic dysfunction in CFS.

Materials and methods

Subjects. Patients were recruited through three Italian referral Centres for CFS (Oncologic Reference Center, Aviano, Professor U. Tirelli; Department of Infectious Diseases, University of Chieti, Professor E. Pizzigallo; Department of Rheumatology, University of Pisa, Dr L. Bazzichi). Only patients with a certified diagnosis of CFS were enrolled. A questionnaire was used to re-confirm the diagnosis and to exclude any other possible causes of fatigue based on symptom referral, lab tests and personal/family histories of immune or hereditary disease. A biological bank of peripheral blood, mRNA, serum and red blood cells from CFS patients was established at the Department of Genetics and Microbiology, University of Pavia, as previously described (7).

The controls comprised 217 healthy individuals recruited in Italy whose immunogenetic data have been reported in the literature (21).

Approval for the study was obtained from the Ethics Committee of the University of Pavia.

KIR typing. In 46 randomly selected patients, KIR molecular typing was conducted using the polymerase chain reaction-sequence specific primers (PCR-SSP) technique with a commercial kit (Pel-Freez, Brown Deer, WI, USA) previously validated in an Italian collaborative study of 21

immunogenetics laboratories (21). PCR amplification parameters were as follows: 1 min at 96°C; 30 cycles of 20 sec at 94°C, 20 sec at 63°C, 90 sec at 72°C; then the temperature was brought to 4°C.

HLA-class I ligand typing. In 44 CFS patients and 50 randomly selected controls, KIR ligand genotyping was conducted using PCR-SSP with a commercial kit (Olerup SSP AB, Hasselstigen, Saltsjobaden, Sweden). Specifically, this kit allowed for the investigation of the presence of the polymorphisms HLA-Cw^{asparagine80}, HLA-Cw^{lysine80}, HLA-B^{Bw4+threonine80}, HLA-B^{Bw4+isoleucine80} and HLA-A^{Bw4+}. PCR amplification parameters were as follows: 2 min step at 94°C; 10 cycles of 10 sec at 94°C, then 60 sec at 65°C; 20 cycles of 10 sec at 94°C, then 50 sec at 61°C, then 30 sec at 72°C; the temperature was then brought to 4°C.

The results were read on ready-to-use precasting gels containing ethidium bromide and 2% agarose under a UV transilluminator.

Statistical analysis. Data analysis was conducted taking into account single KIR genes, KIR genotypes, HLA-ligand motifs and KIR-HLA ligands in CFS patients and controls. Differences were evaluated by the χ^2 or Fisher's exact test. Bonferroni correction was also applied considering the number of independent comparisons performed, and the corrected p-value (p_c) was calculated. Two-point linkage disequilibrium among KIR genes was also considered, and the degree of linkage disequilibrium was expressed with D' statistics measuring the standard deviation from a random association between two alleles at different loci, calculated from the difference (Δ) between the observed haplotypic frequency and the expected frequency given by the product of the observed allelic frequencies of the two markers: $\Delta = p_{ij} - p_i p_j$, where p_{ij} is the frequency of the haplotype having the allele i at the first locus and the allele j at the second locus, and p_i and p_j are the frequencies of alleles i and j respectively. R was obtained as a ratio between Δ and D_{\max} ; the maximum value Δ reached given p_i and p_j .

Results

Prevalence of KIR3DS1 carriers among CFS patients. The distribution of 16 KIR genes in 46 Italian CFS patients and in 217 Italian controls is shown in Table I. The activating KIR genes were labelled with an 'S', which stands for short intracellular tail, while the inhibitory KIR genes were labelled with an 'L', which stands for long intracellular tail. Control data were obtained through a national collaborative network in which our group has also participated, and have been published previously (21). We confirmed the presence of the framework KIR2DL4, KIR3DL2 and KIR3DL3 genes in all patients and in 96-100% of controls.

In terms of the gene content (the presence or absence of one or more KIR genes), the percentage of carriers of more than three activator KIR genes was generally higher in CFS patients (45.7%) than in controls (35.6%), though the difference was not significant. The frequency of patients carrying the KIR3DS1 gene was significantly increased with respect to the controls (54.3 vs. 35.4%; OR=2.16, $p=0.017$), although this

Table I. Distribution of KIR gene carriers in CFS patients and healthy controls.

KIR genes	CFS (n=46)	Controls (n=217)	p-value	OR	95% CI
KIR2DL1	46 (100%)	208 (95.9%)	NS		
KIR2DL2	24 (52.2%)	115 (53.0%)	NS		
KIR2DL3	44 (95.7%)	192 (88.5%)	NS		
KIR2DL4	46 (100%)	216 (99.5%)	NS		
KIR2DL5	26 (56.5%)	106 (48.8%)	NS		
KIR3DL1	43 (93.5%)	210 (96.8%)	NS		
KIR3DL2	46 (100%)	217 (100%)	NS		
KIR3DL3	46 (100%)	216 (99.5%)	NS		
KIR2DS1	20 (43.5%)	79 (36.4%)	NS		
KIR2DS2	25 (54.3%)	115 (53.0%)	NS		
KIR2DS3	14 (30.4%)	71 (32.7%)	NS		
KIR2DS4	43 (93.5%)	210 (97.2%)	NS		
KIR2DS5	12 (26.1%)	61 (28.1%)	NS		
KIR3DS1	25 (54.3%)	77 (35.5%)	0.017	2.16	1.137-4.119

CFS, Chronic fatigue syndrome; NS, not significant.

result was not significant after Bonferroni correction (p_c =NS). Nevertheless, there was a trend indicating an association between the KIR3DS1 gene and CFS. Thus, we decided to further examine the genotypes.

Prevalence of the genotype bearing all but the KIR2DS5 gene in CFS patients. The presence/absence of the KIR genes was analysed in CFS patients with respect to the controls. To date, almost 40 KIR genotypes (excluding pseudogenes) have been described in the literature. A detailed list of genotype profiles present in both Italian patients and controls, using the numerical classification adopted by Uhrberg in 2002 for Caucasian populations (22), is shown in Table II. The last line, defined as 'other', comprises the unique profiles found in our sample, which have yet to be assigned a classification number as they have not yet been described in the literature.

The CFS patients presented 19 different genotype combinations whose haplotypes were not unambiguously assessed as family analysis was not undertaken. Of these, 10 have previously been described in Caucasian populations and 7 were found in Italian controls; however, 9 CFS patients and 29 control genotypes have yet to be described in the literature. Using a comparative analysis of KIR genotype frequency, genotype number 7, which contains all but the KIR2DS5 gene, was observed to be significantly more frequent in patients with respect to the controls: 15.2 vs. 3.7% (OR=4.69, p =0.003, p_c =0.038).

Negative linkage disequilibrium between KIR3DS1 and KIR2DL5 or KIR2DS5. Two-point linkage analysis between different KIR genes in patients revealed similar results to the controls described in a report by Bontadini *et al*, and confirms the presence of linkage disequilibrium between certain KIR genes (21). This makes the evaluation of the independent contribution of single KIR genes towards susceptibility or protection from a disease more difficult. The most interesting

data are the Δ values for the KIR3DS1 gene, which was in positive linkage disequilibrium with KIR2DS1 (Δ =0.012; R =0.039; H =0.248; p =0.001; p_c =0.04), as in the control population, but in negative linkage disequilibrium with KIR2DL5 (Δ =-0.014; R =-0.046; H =0.293; p =0.005) and KIR2DS5 (Δ =-0.001; R =-0.004; H =0.140; p =0.020), in contrast to the controls and contrary to the results expected, since the KIR3DS1, KIR2DL5 and KIR2DS5 genes map very closely in the telomeric part of the KIR region on the long arm of chromosome 19 (23). However, these results were not significant after Bonferroni correction.

No differences between HLA ligand distribution in CFS patients and controls. In the HLA class I molecules, there is a dimorphism that causes a different ligand affinity for different KIR receptors. This dimorphism is located at residue 80, where isoleucine may be replaced by threonine in the HLA-B molecules and asparagine may be substituted by lysine in the HLA-C dimers. Here, the distribution of the HLA amino acid motifs in 44/46 Italian CFS patients and in 50/217 controls was analysed, in particular the presence of HLA-Cw^{asparagine80} (group C1), HLA-Cw^{lysine80} (group C2), HLA-B^{Bw4+threonine80}, HLA-B^{Bw4+isoleucine80}, HLA-A^{Bw4+isoleucine80}, Bw4 supertype, all but A-shared Bw4 motifs and isoleucine 80 alone. The distribution of HLA ligand motifs showed no statistically significant difference between patients and controls.

Excess of ligand-free KIR3DS1 and KIR3DL1 in CFS patients. The interaction between HLA ligands and KIR receptors was examined. KIR2DL1 and KIR2DS1 interact with HLA-Cw^{lysine80}, while KIR2DL2, KIR2DL3 and KIR2DS2 interact with the HLA-Cw^{asparagine80} amino acid motif (24). KIR3DL1 and KIR3DS1 interact with HLA-B^{Bw4+} (25), while KIR3DL2 interacts with some HLA-A sequence motifs (26). The various combinatorial frequencies of KIRs with their ligands were determined. Significant results were obtained

Table II. KIR genotype frequencies in CFS patients and a control Italian population.

KIR genotype											CFS patients		Controls	
	2	2	2	2	2	2	3	3	3	3	N	%	N	%
1	+	-	+	+	-	-	+	+	+	-	13	28.3	61	28.1
2	+	+	+	+	-	+	+	+	+	-	4	8.7	28	12.9
3	+	+	+	+	+	-	+	+	+	-	2	4.3	20	9.2
4	+	-	+	+	+	-	+	+	+	+	4	8.7	18	8.3
5	+	+	+	+	+	+	+	+	+	-	0	0.0	8	3.7
6	+	+	+	+	+	+	+	+	+	+	1	2.2	10	4.6
7	+	+	+	+	+	+	+	+	+	+	7	15.2	8	3.7 ^a
9	+	+	-	+	+	+	+	+	+	+	0	0.0	4	1.8
10	-	+	+	+	-	+	+	+	+	-	0	0.0	2	0.9
11	+	-	+	+	+	-	+	+	+	+	0	0.0	3	1.4
12	+	+	+	+	+	+	+	+	+	+	2	4.3	10	4.6
13	+	+	+	+	+	+	+	+	+	+	1	2.2	0	0.0
14	+	-	+	+	+	-	+	+	+	+	0	0.0	1	0.5
15	+	+	+	+	-	-	+	+	+	-	0	0.0	1	0.5
18	+	+	-	+	+	+	+	+	+	+	0	0.0	1	0.5
19	+	+	+	+	+	+	+	+	+	-	0	0.0	2	0.9
20	+	-	+	+	+	+	+	+	+	-	0	0.0	2	0.9
23	-	+	+	+	+	+	+	+	+	+	0	0.0	1	0.5
25	+	+	-	+	+	+	+	+	+	+	0	0.0	1	0.5
26	+	-	+	+	+	-	+	+	+	-	0	0.0	3	1.4
30	+	+	+	+	+	+	+	+	+	+	0	0.0	2	0.9
34	+	+	+	+	+	+	+	+	+	-	0	0.0	1	0.5
35	+	+	+	+	+	+	+	+	+	+	2	4.3	0	0.0
36	+	-	+	+	+	-	+	+	+	-	1	2.2	0	0.0
40	-	+	+	+	-	+	+	+	+	-	0	0.0	1	0.5
Other											9	19.6	29	13.7
Total											46		217	

^ap=0.003, OR=4.69, 95% CI 1.607-13.677.

Table III. KIR3DL1/3DS1 and HLA cognate ligands in CFS and in controls.

KIR	HLA KIR ligand	CFS (n=44)	Controls (n=50)	p-value	OR	95% CI
3DL1	Bw4 ^{Ile80}	16 (36.3%)	26 (52%)	NS		
3DL1	without Bw4 ^{Ile80}	25 (56.8%)	21 (42%)			
3DS1	Bw4 ^{Ile80}	7 (15.9%)	12 (24%)	NS		
3DS1	without Bw4 ^{Ile80}	17 (38.6%)	11 (22%)			
3DL1	Bw4 ^{Thr80}	12 (27.3%)	12 (24%)	NS		
3DL1	without Bw4 ^{Thr80}	29 (66.0%)	35 (70%)			
3DS1	Bw4 ^{Thr80}	9 (20.4%)	4 (8%)	NS		
3DS1	without Bw4 ^{Thr80}	15 (34.0%)	19 (38%)			
3DL1	A ^{Bw4Ile80}	15 (34.0%)	21 (42%)	NS		
3DL1	without A ^{Bw4Ile80}	26 (59.0%)	26 (52%)			
3DS1	A ^{Bw4Ile80}	8 (18.0%)	15 (30%)			
3DS1	without A^{Bw4Ile80}	16 (36.0%)	8 (16%)	0.023	3.00	1.132-7.946
3DL1	Ile80 motif	23 (52.3%)	33 (66%)			
3DL1	without Ile80 motif	35 (79.5%)	26 (52%)	0.005	3.59	1.432-8.990
3DS1	Ile80 motif	12 (27.3%)	19 (38%)			
3DS1	without Ile80 motif	22 (50.0%)	11 (22%)	0.004	3.54	1.452-8.657

CFS, Chronic Fatigue Syndrome; NS, not significant.

regarding KIR3DS1/3DL1 and their HLA cognate ligands (Table III). The frequency of individuals with a KIR3DS1 receptor free from its HLA-A^{Bw4+Ile80} ligand was increased in the CFS group compared to the controls (36 vs. 16%; $p=0.024$, $OR=3.0$, $p_c=NS$). The frequency of KIR3DL1 receptors missing of their HLA-ligand (containing the Ile80 motif) was also significantly higher in CFS patients than in controls (79.5 vs. 52%; $p=0.005$, $OR=3.59$, $p_c=0.04$). Moreover, a significantly increased frequency of individuals with KIR3DS1 lacking their cognate HLA Ile80 motif was observed in the CFS group with respect to the controls (50 vs. 22%; $p=0.004$, $OR=3.55$, $p_c=0.032$).

Discussion

The KIR gene system consists of a family of polymorphic genes (16 functional and 2 pseudogenes) which map on chromosome 19q13.4 inside a 1Mb region known as the leukocyte receptor complex (LRC). Associations between the presence of certain KIR genes and diseases such as rheumatoid arthritis, psoriatic arthritis, scleroderma and p-ANCA positive vasculitis have been described (27-30). It is, however, their specific interaction with HLA ligands that explains their potential involvement in the occurrence of immune-mediated disease, in cancer progression, in the clearance of a pathogen, or in transplant engraftment. Through the interaction between KIR-HLA, NK cells represent the link between innate and adaptive (31).

Based on the results of the present study, we hypothesize that the presence of the activating gene KIR3DS1 may confer susceptibility to CFS. This appears to support a scenario in which the risk of developing CFS is, through a series of events, dependent on the level of NK cell activation.

KIR3DS1 is considered to belong to the lineage of KIR3DL1, and HLA-Bw4 is its ligand. KIR3DS1 is included in the 'B haplotype' and excluded from the 'A haplotype'. It is known that while the KIR A haplotype has a relatively homogeneous genetic organization, characterized by a high degree of inhibitory genes, the B haplotype is much more variable and contains a greater number of activating genes (36). More activating KIR genes appear to be present in CFS patients: 45.7% of patients possess more than three activating KIR genes, compared to 35.6% of controls. Therefore, if we consider the KIR profile alone (i.e., without ligand), it is reasonable to argue that this syndrome is caused by insufficient inhibitory regulation, as has been hypothesized in certain autoimmune diseases, such as insulin-dependent diabetes mellitus (32). A simple interpretation would suggest that KIR3DS1 stimulation of the production of inflammatory cytokines by NK cells contributes to ignite and sustain an inflammatory condition (33).

However, the NK cells in CFS are rather anergic, showing a very low degree of both inhibitory and activating activity (34). The key to interpretation may be in the KIR/HLA interaction rather than in one genetic system alone. In effect, CFS patients seem to present an excess of KIR3DL1 and KIR3DS1 receptors free from their HLA-Bw4^{isoleucine80} ligand, suggesting at least two possible scenarios: i) the disease may depend on the presence of the KIR3DL1 inhibitory receptor in the absence of its corresponding ligand, a condition that generates a serious imbalance in the immune response towards stressful events; or ii) the lack of KIR3DL1/KIR3DS1 cognate ligands may be responsible for the low activity of NK cells, which has been demonstrated in patients and is explained here (19,34).

This study was limited by the small patient sample, due mainly to the difficulties in positively diagnosing CFS. The diagnosis of CFS is based on a constellation of symptoms and

clinical indicators, as no pathognomonic tests are available, and only few medical centres in Italy are capable of diagnosing it. However, our sample size was appropriately powered based upon the observed frequency in our control population.

This study may help elucidate the pathogenesis of CFS. The findings indicate that CFS may be caused by a partially deficient interaction between innate (KIR) and adaptive (HLA) immunity.

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