Killer Cell Immunoglobulin-like Receptor Genotype and Haplotype Investigation of Natural Killer Cells from an Australian Population of Chronic Fatigue Syndrome/Myalgic Encephalomyelitis Patients

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ABSTRACT: *Killer cell immunoglobulin-like receptor (KIR)* genes encode for activating and inhibitory surface receptors, which are correlated with the regulation of Natural Killer (NK) cell cytotoxic activity. Reduced NK cell cytotoxic activity has been consistently reported in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) patients, and *KIR* haplotypes and allelic polymorphism remain to be investigated. The aim of this article was to conduct a pilot study to examine *KIR* genotypes, haplotypes, and allelic polymorphism in CFS/ME patients and nonfatigued controls (NFCs). Comparison of *KIR* and allelic polymorphism frequencies revealed no significant differences between 20 CFS/ME patients and 20 NFCs. A lower frequency of the telomeric A/B motif ($P < 0.05$) was observed in CFS/ME patients compared with NFCs. This pilot study is the first to report the differences in the frequency of *KIR* on the telomeric A/B motif in CFS/ME patients. Further studies with a larger CFS/ME cohort are required to validate these results.

KEYWORDS: Natural Killer cells, Chronic Fatigue Syndrome/Myalgic Encephalomyelitis, *Killer cell immunoglobulin-like receptor,* haplotype, cytotoxic activity

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Introduction

Natural Killer (NK) cells are effector cells of the innate immune system, and following recognition of a potential target cell, NK cells mediate a response through cytotoxic activity to remove the target cells or cytokine production to direct an immune response.[1] Activation of NK cell cytotoxic activity is a tightly regulated process governed by the balance of signals received from surface receptors.[2] NK cells constitutively express a myriad of surface receptors, which can be structurally grouped into the immunoglobulin superfamily and the C-type lectin family.[2,3] One major family of NK cell receptors includes the Killer cell immunoglobulin-like receptors (KIRs).[2,3] Through *KIR* receptors, NK cells recognize target cells with reduced or absent expression of human leukocyte antigen, which may be the result of infection, malignant transformation, or cellular stress.[4] KIR engagement with specific human leukocyte antigen ligands transduces a cascade of signals to inhibit or activate NK cell cytotoxic activity.[2]

KIRs expressed on NK cells are encoded by 17 *KIRs*, which are located on human chromosome 19q13.4 in the leukocyte receptor cluster.[5] Of the 17 *KIRs* characterized, nine genes encode inhibitory receptors (*KIR3DL3*, *KIR2DL2*, *KIR2DL3*, *KIR2DL5B*, *KIR2DL1*, *KIR2DL4*, *KIR3DL1*, *KIR2DL5A,* and *KIR3DL1*), six encode activating receptors (*KIR2DS2*, *KIR2DS3/2DS5C*, *KIR3DS1*, *KIR2DS3/2DS5T*, *KIR2DS4*, and *KIR2DS1*), and the remaining two are pseudogenes (*KIR2DP1* and *KIR3DP1*) with unknown functions.[4] Within the leukocyte receptor cluster, the *KIR* locus is defined by conserved framework genes including *KIR3DL3*, *3DP1*, *2DL4*, and *3DL2*, which also mark centromeric and telomeric regions.[6] *KIRs* in the centromeric and telomeric regions are genetically diverse due to variability in gene content and allelic polymorphisms.[7,8] The combination of *KIR* and pseudo genes gives rise to a number of different genotypes, which according to the presence or absence of specific *KIRs* can be further classified as haplotypes A or B.[6] Haplotype A predominantly consists of inhibitory genes including *KIR2DL1*, *KIR2DL3*, *KIR3DL1*, and *KIR3DL2* and the activating *KIR2DS4*.[5] *KIR* haplotypes that do not contain the exact copy of haplotype A genes are classified as haplotype B.[5] The predominance of inhibitory genes in haplotype A and activating genes in haplotype B suggests a distinct role of KIR

haplotypes in governing effector functions of NK cells.[9] *KIR* association studies have suggested that haplotype A provides more effective immunity for the clearance of viral infections including hepatitis C and Ebola compared with haplotype B due to the regulation of NK cell activity.[10–12] *KIR* haplotypes present on the centromeric or telomeric motifs are also known to influence NK cell function.[5,6] For example, haplotype B on centromeric and telomeric motifs has been identified to provide protection against relapse in hematopoietic stem cell transplantation.[5] In kidney transplant patients, B haplotypes on the telomeric motif has been suggested to protect against cytomegalovirus infection.[5,6]

Variations in *KIR* gene content and allelic polymorphism have been identified to influence KIR surface expression and receptor ligation required to initiate NK cell cytotoxic activity and cytokine production.[12–16] Reduced NK cell cytotoxic activity has consistently been reported in patients with Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME).[17–25] While one study has identified that CFS/ME patients have increased frequencies of *KIR3DS1*, additional levels of genetic diversity including KIR haplotypes, centromeric and telomeric haplotypes, and allelic polymorphism, which may contribute to reduced NK cell cytotoxic activity, remain to be investigated.[26] The aim of this pilot study was to investigate *KIR* genotypes, haplotypes, and allelic polymorphism in CFS/ME patients and nonfatigued controls (NFCs).

Materials and Methods

Study participants and inclusion criteria. CFS/ME patients and NFCs were recruited from a database at the National Centre for Neuroimmunology and Emerging Diseases, Menzies Health Institute Queensland, Australia. In the absence of a diagnostic test, the 1994 Fukuda definition was used to identify CFS/ME patients.[27] All participants completed an online questionnaire based on the Fukuda definition for fatigue and symptom presentation to determine suitability for study inclusion. Exclusion criteria included participants presenting with primary mood disorders, thyroid conditions, diabetes, epilepsy, psychosis, cardiac disorders, smoking, pregnant or breastfeeding, and immunological, inflammatory, or autoimmune diseases.

Compliance with ethical standards. Written informed consent was obtained from all participants. This study was conducted with the approval of the Griffith University Human Research Ethics Committee (MSC22/12/HREC) and in accordance with the ethical standards of the 1964 Declaration of Helsinki.

Blood collection. Thirty-five milliliters of peripheral blood was collected into ethylenediaminetetraacetic acid tubes from the antecubital vein of each participant. Blood samples were collected between the hours of 7:30–10:00 am to eliminate circadian variation and analyzed within four hours of collection.[28] Participant blood parameters including full blood counts of white and red blood cells, electrolytes, and erythrocyte sedimentation rate were assessed on all samples by Pathology Queensland.

NK cell isolation and DNA extraction. Peripheral blood mononuclear cells were isolated by density gradient centrifugation with Ficoll-Hypaque (GE Healthcare). From the peripheral blood mononuclear cells, NK cells were isolated by a negative selection kit according to the manufacturer's instructions (Miltenyi Biotec). Isolated NK cells were frozen in liquid nitrogen and stored for deoxyribonucleic acid (DNA) extraction at a later date. DNA from NK cells was extracted using the QIAamp DNA extraction kit (Qiagen) according to manufacturer's instructions, and the concentration and quality of each DNA extraction was assessed using the NanoDrop Spectrophotometer 1000 (NanoDrop Technologies). Prior to genetic typing, NK cell DNA was stored at –20 °C.

***KIR* gene content.** *KIR* genotyping was performed using reagents and software at Scisco Genetics.[6,29,30] Briefly, 14 locus-specific primer pairs were used for the initial polymerase chain reaction (PCR) amplification to detect *KIR3DL3*, *2DS*, *2DL2*, *2DL3*, *2DL5B*, *2DS3/DS5C*, *2DP1*, *2DL1*, *3DP1*, *2DL4*, *3DL1*, *3DS1*, *2DL5A*, *2DS3/2DS5T*, *2DS1*, *2DS4*, and *3DL2*.[29] PCR amplicons generated from each individual were pooled and treated with Exonuclease I and alkaline phosphatase. The amplicon targets were then combined with DNA linkers containing adaptor sequences, which served as primer-binding sites for dual-indexing barcode PCR to ensure unique identification of each sample. Following barcoding, the samples were pooled and multiplex sequencing was performed using the MiSeq platform (Illumina). The generated sequencing data were aligned to sequences obtained from the Immuno Polymorphism-KIR Database to determine *KIR* and allelic assignments for each participant.[31] Participants were also stratified according to the number of activating (1–6) and inhibitory (6–9) *KIRs* present.

KIR haplotypes. KIR haplotypes in CFS/ME patients and NFCs were identified according to the presence or absence of specific *KIRs*. Haplotype A was determined according to the presence of nine *KIRs: 3DL3*, *2DL3*, *2DP1*, *2DL1*, *3DP1*, *2DL4*, *3DL1*, *2DS4*, and *3DL2*.[6] Haplotype B was identified according to the absence of all haplotype A genes.[6] Participants presenting with only haplotype A genes were assigned as A/A genotype, homozygous participants for haplotype B were assigned as B/B, and heterozygous individuals containing haplotype A and B genes were assigned as A/B.[32]

Centromeric and telomeric motif KIR haplotypes. The position of *KIRs* within the KIR locus can further define the centromeric and telomeric motifs as genotypes A/A, B/B, or A/B.[6] Haplotype A *KIR* on the centromeric motif includes *KIR3DL3*, *2DL3*, *2DP1*, *2DL1*, and *3DP1*, while *2DL4*, *3DL1*, *2DS4*, and *3DL2* are found on the telomeric motif. Centromeric and telomeric motifs with only haplotype B genes were assigned B/B, and participants with a combination of haplotype A and B genes on both motifs were classified as A/B.

Statistical analysis. Statistical analysis of the data was performed on the Statistical Package for the Social Sciences (IBM Corp, Version 22). For routine blood parameters, Shapiro–Wilk test was used to test for Gaussian distribution. The independent Mann–Whitney test was used to identify any significant differences in blood parameters between CFS/ME patients and NFCs. Frequencies of *KIRs*, haplotypes, centromeric and telomeric haplotypes, and *KIR* alleles were compared between CFS/ME patients and NFCs using Fisher's test of association (for frequency counts less than five) and the chi-square test (for frequency counts greater than five). *P*-values of <0.05 were considered statistically significant.

Results

Participants, blood parameters, and NK cell purity. All participants were Caucasian and a total of 20 CFS/ME patients meeting the 1994 Fukuda definition (mean age [years] ± standard error of the mean = 53.2 ± 2.26) and 20 NFCs (mean age [years] ± standard error of the mean = 52.85 ± 1.70) were included in this study. No significant differences were observed when the ages, white and red blood cell parameters, electrolytes, C-reactive protein, and erythrocyte sedimentation rate were compared between CFS/ME patients and NFC participants (Supplementary Table 1). The mean purities of isolated $CD56^{+}CD3^{-}$ NK cells for CFS/ME patients and NFCs were 98.0% and 98.9%, respectively.

Frequency of activating and inhibitory KIRs present in CFS/ME patients. No significant differences were observed in the frequency of activating and inhibitory genes between CFS/ME patients and NFCs (Fig. 1). The frequency of two and five activating genes (A) and six and seven inhibitory genes (B) was higher in CFS/ME patients compared with NFCs, although this difference was not significant (B).

No significant difference in *KIR* gene frequencies in CFS/ME patients. The frequency of individual activating and inhibitory *KIRs* was compared between CFS/ME patients and NFCs, and no significant differences were observed (Fig. 2). Although not significant, frequency of the activating *KIR2DS2* was higher in CFS/ME patients when compared with the NFCs (A). For *KIR2DS3/2DS5C*, *KIR3DS1*, *KIR2DS3/2DS5CT*, *KIR2DS1*, and *KIR2DS4*, the frequency was lower in CFS/ME patients compared with the NFCs. Inhibitory *KIRs*, namely, *KIR3DL3*, *KIR2DL1*, *KIR2DL4*, and *KIR3DL2*, were present in all CFS/ME patients and NFCs (B). In CFS/ME patients, the frequency of *KIR2DL2* and *KIR2DL3* was higher compared with NFCs. The frequency of *KIR2DL5B*, *KIR3DL1*, and *KIR2DL5A* was lower in CFS/ME patients compared with NFCs, although these differences were not significant.

Table 1. Distribution of *KIR* genotype including centromeric and telomeric motifs in CFS/ME patients and NFCs ($^{*}P < 0.05$).

	CFS/ME n = 20 (%)	NFC n = 20(%)	*P* VALUE	OR	95% CI
Genotype					
A/A	4 (20)	4 (20)	1.000	1.000	0.212–4.709
B/B	1 (5)	0 (0)	1.000	–	–
A/B	15 (75)	16 (80)	1.000	1.333	0.300–5.926
Centromeric motif					
A/A	7 (35)	10 (50)	0.337	1.857	0.522–6.612
B/B	4 (20)	1 (5)	0.342	0.211	0.021–2.079
A/B	9 (45)	9 (45)	1.000	1.000	0.288–3.476
Telomeric motif					
A/A	12 (60)	8 (40)	0.206	0.444	0.125–1.575
B/B	3 (15)	0 (0)	0.231	–	–
A/B	**5 (25)**	**12 (60)**	***0.025**	4.500	1.166–17.373

Note: Data are presented as *n* (frequency % within group).
Abbreviations: CI, confidence interval; OR, odds ratio.

Telomeric A/B haplotype motif associated with CFS/ME patients. CFS/ME and NFC participants were classified as A/A, B/B, or A/B genotypes according to the presence or absence of specific *KIRs*, and no significant differences were observed (Table 1). A lower frequency of the A/B telomeric motif was observed in CFS/ME patients ($P < 0.05$) compared with NFCs.

Frequency distribution of KIR alleles in CFS/ME patients. The presence of alleles associated with activating

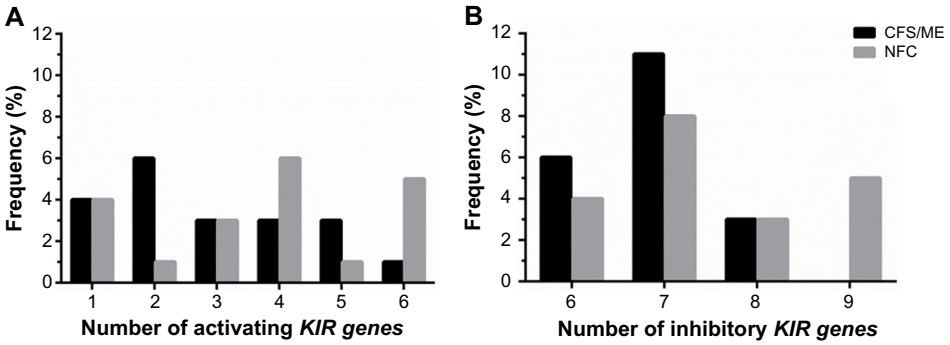

Figure 1. Frequency of activating (**A**) and inhibitory (**B**) *KIRs* present in CFS/ME patients and NFCs. Depending on the *KIRs* detected in each individual, participants were stratified according to the number of activating (1–6) and inhibitory (6–9) *KIRs* present. Data are presented as the frequency of each number of genes present within CFS/ME patients or NFCs. No significant differences were observed.

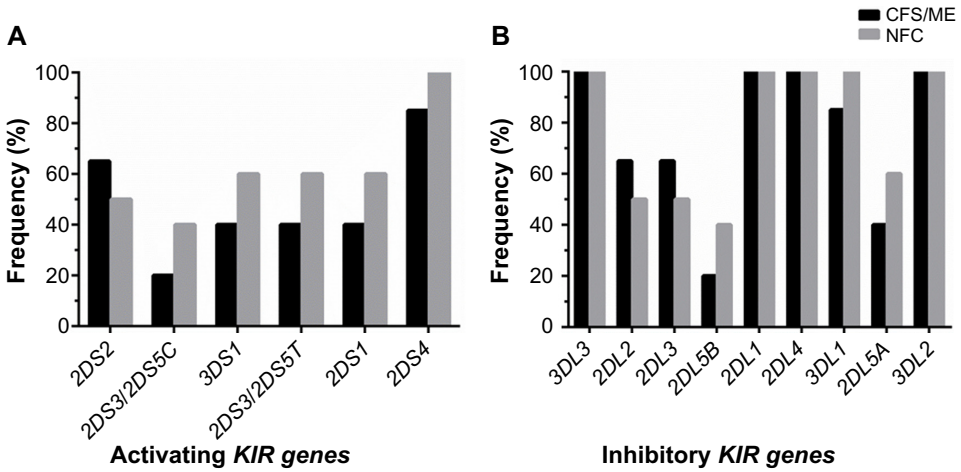

Figure 2. Frequency distribution of activating *KIRs* (**A**) and inhibitory *KIRs* (**B**) in CFS/ME patients and NFCs. For each participant, sequencing data generated was aligned to sequences obtained from the Immuno Polymorphism-KIR Database to determine the presence of each *KIR*. Data are presented as the percentage of each gene present within CFS/ME or NFCs, and no significant differences were observed between the two groups.

and inhibitory *KIRs* was compared between CFS/ME and NFC participants. While CFS/ME patients presented with increased frequencies of *KIR2DS2**007 and lower frequencies of *KIR3DS1**014 and *KIR2DL3**00110 compared with NFCs, no significant differences were observed (Table 2).

Discussion

This pilot study is the first to genotype NK cell *KIRs* in an Australian population of CFS/ME and also the first to investigate *KIR* haplotype frequencies in CFS/ME patients. *KIRs* encode for activating and inhibitory surface receptors, which have previously been correlated with the regulation of NK cell cytotoxic activity.[2,13,33–35] Reduced NK cell cytotoxic activity has been consistently reported in CFS/ME patients, and investigation of *KIRs* in the present study has revealed a significantly lower frequency of the telomeric A/B motif in CFS/ME.

The numbers of activating and inhibitory *KIRs* present were compared between CFS/ME patients and NFCs as gene quantity has been associated with NK cell activation.[36,37] While differences were reported in the number of activating and inhibitory genes between CFS/ME patients and NFCs, statistical significance was not observed. Specific *KIRs* were also examined and no significant differences were reported between CFS/ME patients and NFCs. These findings contrast a previous association report of increased activating *KIR3DS1* in CFS/ME patients.[26] An increased frequency of *KIR3DS1* has been correlated with increased NK cell degranulation and production of interferon-gamma.[38] Previously, we have also reported increased degranulation and interferon-gamma production in NK cells from CFS/ME patients, which suggests that frequencies of *KIR3DS1* may contribute to NK cell dysfunction in CFS/ME.[18,26]

Inherited diversity of *KIR* genotypes through the combination of maternal and paternal haplotypes on the centromeric and telomeric motifs has been associated with susceptibility or resistance to pathogen infection due to the regulation of NK cell activity.[39] Within the telomeric motif of the *KIR* locus, CFS/ME patients presented with a lower frequency of the A/B genotype compared with the NFCs. More than half of the CFS/ME cohort presented with homogenous A/A telomeric motif, which only contains one activating receptor, *KIR2DS4*. Due to the predominance of inhibitory *KIRs* in homogenous A/A genotypes, ligation of KIR2DL3, KIR2DL1, and KIR3DL1confers strong inhibition through the immunoreceptor tyrosine-based inhibitory motifs.[2,40] In contrast, inhibition of NK cells in haplotype B individuals are mediated by fewer ligands due to the absence of these inhibitory genes.[40] Differences in the presence of activating and inhibitory *KIRs* between A, B, and AB suggests that each haplotype may have different activation thresholds for NK cells, which may be dysfunctional in CFS/ME patients.

In addition to the *KIR* content variation between the haplotypes, allelic polymorphism caused by insertions, deletions, substitutions, or single-nucleotide polymorphisms also contributes to the regulation of NK cytotoxic activity.[14,16,41–43] The inhibitory function of NK cells is affected by substitutions of *KIR3DL1* producing *KIR3DL1**004, *KIR3DL1**002, and *KIR3DL1**007.[14,32] *KIR3DL1**004 results in the production of a misfolded protein, which is retained in the endoplasmic reticulum, while *KIR3DL1**002 transduces a stronger inhibitory response than *KIR3DL1**007 due to conformational changes in the extracellular region of the receptor.[16] As allelic polymorphisms have been associated with changes in the levels of KIR surface expression and strength of signals integrated due to ligand affinity, *KIR* alleles were investigated in CFS/ME patients and no significant differences were observed.

Table 2. Frequency distribution of KIR alleles in CFS/ME patients and NFCs.

GENE	ALLELE	CFS/ME n = 20 (%)	NFC n = 20 (%)	*P* VALUE	OR	95% CI
Activating genes						
KIR2DS2	*00104	10 (50)	8 (40)	0.751	0.674	0.159–2.764
	*007	7 (35)	3 (15)	0.273	0.337	0.047–1.840
	*008	2 (10)	1 (5)	1.000	0.482	0.008–10.024
KIR2DS3	*004	5 (25)	8 (40)	0.501	1.965	0.431–9.833
	*005	1 (5)	1 (5)	1.000	1.000	0.012–82.524
KIR2DS5	*003	8 (40)	11 (55)	0.527	1.805	0.442–7.726
	*012	8 (40)	9 (45)	1.000	1.221	0.294–0.518
KIR3DS1	*014	5 (25)	9 (45)	0.320	2.399	0.537–11.930
	*049N	1 (5)	2 (10)	1.000	2.073	0.100–130.885
	*055	1 (5)	0 (0)	1.000	–	–
	*078	1 (5)	0 (0)	1.000	–	–
KIR2DS1	*001	1 (5)	0 (0)	1.000	–	–
	*008	11 (55)	13 (65)	0.748	1.504	0.357–6.571
KIR2DS4	*00102	3 (15)	1 (5)	0.605	0.307	0.005–4.243
Inhibitory genes						
KIR3DL3	*047	0 (0)	1 (5)	1.000	–	–
	*054	0 (0)	1 (5)	1.000	–	–
	*056	0 (0)	1 (5)	1.000	–	–
KIR2DL2	*00303	7 (35)	4 (20)	0.480	0.473	0.082–2.362
	*013	2 (10)	1 (5)	1.000	0.482	0.008–10.024
KIR2DL3	*016	17 (85)	18 (90)	1.000	1.570	0.159–20.979
	*00110	4 (20)	9 (45)	0.176	3.174	0.672–17.894
	*020	4 (20)	2 (10)	0.661	0.453	0.036–3.663
	*015	3 (15)	4 (20)	1.000	1.404	0.202–11.128
	*011	2 (10)	1 (5)	1.000	0.482	0.008–10.024
	*030	1 (5)	2 (10)	1.000	2.073	0.100–130.885
KIR2DL1	*023	13 (65)	13 (65)	1.000	1.000	0.225–4.451
	*022	3 (15)	1 (5)	0.605	0.307	0.005–4.243
	*021	2 (10)	1 (5)	1.000	0.482	0.008–10.024
	*020	1 (5)	2 (10)	1.000	2.073	0.100–130.885
	*025	1 (5)	1 (5)	1.000	1.000	0.012–82.524
	*026	1 (5)	0 (0)	1.000	–	–
	*008	1 (5)	0 (0)	1.000	–	–
	*009	0 (0)	1 (5)	1.000	–	–
	*00601	1 (5)	1 (5)	1.000	1.000	0.012–82.524
	*00402	0 (0)	1 (5)	1.000	–	–
Inhibitory genes						
KIR2DL4	*0104	3 (15)	0 (0)	0.231	–	–
	*01201	1 (5)	0 (0)	1.00	–	–
	*017	1 (5)	0 (0)	1.00	–	–
	*0080102	0 (0)	1 (5)	1.000	–	–
KIR3DL1	*077	18 (90)	18 (90)	1.000	1.000	0.066–15.205
	*008	3 (15)	1 (5)	0.605	0.307	0.005–4.243
	*033	3 (15)	2 (10)	1.000	0.637	0.048–6.293
	*068	2 (10)	1 (5)	1.000	0.482	0.008–10.024

(*Continued*)

Table 2. (*Continued*)

GENE	ALLELE	CFS/ME n = 20 (%)	NFC n = 20 (%)	*P* VALUE	OR	95% CI
	*072	2 (10)	1 (5)	1.000	0.482	0.008–10.024
	*075	1 (5)	2 (10)	1.000	2.073	0.100–130.885
	*052	1 (5)	0 (0)	1.000	–	–
KIR3DL2	*018	4 (20)	6 (30)	0.716	1.691	0.322–9.940
	*035	3 (15)	3 (15)	1	1.000	0.117–8.570
	*00104	2 (10)	0 (0)	0.487	–	–
	*01302	0 (0)	2 (10)	0.487	–	–
	*056	1 (5)	1 (5)	1.000	1.000	0.012–82.524
	*054	1 (5)	0 (0)	1.000	–	–

Notes: The activating and inhibitory *KIRs* are listed in the first two columns. Depending on the allele detected in each individual, an asterisk (*) signifies the numerical allele designation. Data are presented as *n* (frequency % within group) for participants presenting with each allele.
Abbreviations: CI, confidence interval; OR, odds ratio.

Conclusions

This pilot study is the first to report differences in the frequency of *KIR* on the telomeric A/B motif in CFS/ME patients. As the activity of NK cells is governed by the balance between activating and inhibitory signals, differences in the gene content profile of *KIR* haplotypes may create different activation thresholds for NK cells.[39,40] In CFS/ME patients, further investigations are required to determine if lower frequencies of A/B on the telomeric motif may contribute to dysfunctional regulation of NK cell cytotoxic activity. It is also paramount for future studies to include a larger sample size to ensure that there is enough statistical power to identify the differences between CFS/ME patients and NFCs. Future studies into NK cell *KIRs* have the potential to identify if genetic predispositions may contribute to reduced NK cell cytotoxic activity in CFS/ME patients.

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Author Contributions

Performed all the experimental protocols for NK cell isolation and DNA extraction from NK cells, analyzed the data, and wrote the article: TKH. Helped design the study, analyzed the data, and drafted the article: EWB. Conceived the study, sought ethics approval, provided the CFS/ME, NFC cohorts from the National Centre for Neuroimmunology and Emerging Diseases database, critically revised the intellectual content and interpretation of data analysis, and drafted the article: DRS, SMM-G. All the authors read and approved the final manuscript.

Supplementary Material

Supplementary Table 1. Blood parameters measured in CFS/ME patients and NFC participants.

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