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Low NK Cell Activity in Chronic Fatigue Syndrome (CFS) and Relationship to Symptom Severity

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Abstract

Background: Natural killer (NK) cells act as an immune surveillance against invading pathogens and tumors. NK cell cytotoxicity (NKCC) has been reported to be decreased in patients with CFS.

Methods: The objective of this review was to conduct an analysis of available publications that reported NKCC data in CFS in order to evaluate any relationships to case definitions used to define CFS and symptom severity.

Results: Of 17 studies that evaluated NKCC in patients with CFS, defined using the CDC 1988 and/or 1994 case definition (CD), 88% (15/17) concluded that NKCC was decreased in CFS patients compared to normal controls. The NKCC decrease was seen using two established methods, ^{51}Cr release (11/13) and flow cytometry (4/4). The mean percent decrease in NKCC using the CDC 1988 CD (66.3%) was significantly greater than that using the CDC 1994 CD (49.7%) ($p<0.01$). This result is consistent with that of six publications showing a greater decrease in NKCC associated with increased CFS symptom severity based on the lower symptom requirement for the CDC 1994 vs. 1988 CD. In contrast, there was no significant difference in the mean percent decrease in NKCC seen comparing the CDC 1994 CD defined population using the ^{51}Cr release (48.3%) vs. flow cytometry (50.7%) assays ($p>0.5$). Finally, seven studies investigating the ability of various agents to augment NKCC in patients with CFS showed increases of NKCC with both in vitro exposure (4/5) and in vivo exposure using randomized trials (2/2).

Conclusions: Low NKCC is commonly seen in CFS and is associated with increase symptom severity.

Keywords: NK cells; Cytotoxicity; CFS; NKCC; Illness severity; Case definition

Introduction

Natural killer (NK) cells are an important component of the innate immune response acting as a surveillance mechanism against tumor cells and invading pathogens [1-3]. Recent studies indicate that NK cells can also mount a form of antigen-specific immunologic memory [4]. The importance of NK cells to human health has been shown by the discovery of a growing number of persons who are deficient in NK cells and/or their activity [5]. Patients with deficiencies in NK cell function typically have increased susceptibility to herpes viruses and other viral pathogens, as well as an increased frequency of malignancies [5]. Low NK cell cytotoxicity (NKCC)/low NK cell activity has been reported in individuals with high familial incidences of cancer [2,6].

NKCC has also been reported to be decreased in patients with CFS. CFS is a debilitating disorder characterized by disabling fatigue that is not improved by rest and multiple flu-like symptoms. It is an economically and emotionally devastating condition with an unknown etiology. The prevalence of CFS is estimated to be at least 1 million in the US. CFS occurs most frequently in individuals between 40-50 years of age and is 3 times more prevalent in females. With no approved therapy, current treatment is directed against symptom relief and increased ambulatory function. This review focuses on the reported decrease in NK cell activity as a possible target for development of therapeutic approaches for CFS.

Studies of NK cell number and phenotypes in patients with CFS have been contradictory [7-10]. In contrast, low NKCC has been more consistently reported in CFS patients compared to normal controls [9-13]. When NK cell number versus NKCC was directly compared in CFS, no relationship between these two variables was seen [14]. This review focuses on NK cell activity in CFS comparing results from CFS patient cohorts defined by either the Centers for Disease Control (CDC) 1988 or 1994 case definitions (CD) versus normal controls and as a function of assay method (flow cytometry or ^{51}Cr release from target cells). Included in this review is evidence of an association between decreased NK cell activity and increased CFS symptomatology. Finally, studies examining potential treatments for augmentation of NKCC in CFS are reviewed.

Materials and Methods

The objective of this review was to conduct an analysis of available publications that reported NKCC data in CFS in order to evaluate any relationships to CFS defined using different case definitions and to symptom severity. The literature was searched for publications in English that contained NKCC data from patients with CFS in order to evaluate the percent reduction in NK cell activity compared to normal controls. A search on PubMed was performed for articles with key words “Natural Killer Cell Activity” (NK Cell Activity), “chronic

fatigue syndrome" (CFS), "Flow Cytometry", and "Chromium 51". A comprehensive "Google" search was also performed with the above mentioned keywords for any relevant published articles outside of PubMed. From each published article found, the references in the article were then searched for any additional pertinent information.

Several variables associated with the NKCC data, such as the case definition of CFS utilized, age and gender matching of normal healthy control cohorts, type of NKCC assay utilized, target cell type, effector:target (E:T) cell ratios and lytic units (LU) were evaluated. The goal of LU reporting is to express NKCC independent of arbitrary E:T ratios. A comparison of NKCC results from CFS cohorts defined using the CDC 1988 and 1994 case definitions was performed. Analysis of NK cell activity obtained using the standard chromium (^{51}Cr) release assay versus flow cytometry methodology was also performed. Flow cytometry measures apoptosis using Annexin V – fluorescein isothiocyanate (FITC). Annexin-V binds to phosphatidylserine, normally located in the cytoplasmic side of the cell membrane, which flips to the outer leaflet of the cell membrane at the early phases of apoptotic process, where it can be detected by fluorescent Annexin-V conjugates. If the percent NK cell activity in a reference was only presented graphically in a figure, the number of that figure utilized to derive the NKCC result was noted in the text. The percentage difference in NK cell activity between normal controls and CFS patients was calculated and presented in Tables 1 and 2. Statistical analyses of differences between the percent decrease in NK cell activity in different CFS patient cohorts or between CFS patients and normal controls utilized both the T-test and Wilcoxon-Mann-Whitney test.

Results

Studies of NK cell cytotoxicity/NK cell activity conducted prior to 1988 or not using the CDC 1988/ 1994 CFS case definition

In 1986, Eby et al. investigated a cluster of 8 cases of chronic fatigue in a symphony orchestra containing 67 members [15]. Seven of the 8 cases had been diagnosed with acute mononucleosis or chronic Epstein-Barr Virus (EBV) infection. Mean NKCC using K562 as the target in a ^{51}Cr release assay was 51 lytic units (LU) in the 8 chronically fatigued cases vs. 134 LU in the non-fatigued orchestra members ($p<0.001$). In 1987, low natural killer syndrome (LNKS) was reported in 23 patients with unexplained fever and uncomfortable fatigue in Japan [16] and shared many signs and symptoms with CFS [17]. However, no NKCC results were reported using a cohort of CFS patients defined by the CDC 1988 case definition (CD) [18]. In 1987, Caligiuri, et al. studied 41 patients with chronic fatigue "syndrome" or chronic active EBV infection and 23 healthy controls and categorized each patient's NKCC response compared to the 25th percentile of the asymptomatic control population [19]. Seventy-three percent of the CFS patients (30/41) had reduced NKCC below 33%, the 25th percentile of 23 age and gender matched controls without CFS. Between 1985 and 1987, 26 patients with elevated EBV titers were referred for evaluation of their chronic fatigue [20]. A clinical history of mononucleosis was reported in 14 of the 26 patients. No difference in NK cell activity was seen between these patients and controls. In a retrospective analysis only 6 of the 26 patients fulfilled the CDC 1988 CD for CFS. When these 6 patients were compared with the 20 non-CFS patients, the six had a significantly greater number of symptoms on enrollment (15.8 vs. 9.9, $p<0.01$). There was no discussion of the NKCC values of these 6 patients vs. the 20 patients not fulfilling the CDC 1988 CD.

Studies of NK cell cytotoxicity/NK cell activity utilizing the CDC 1988/1994 CFS case definitions

This review of NKCC and Tables 1 and 2 focus primarily on published studies of CFS that utilized either the CDC 1988 (n=9) or 1994 (n=7) CDs or both (n=1). No studies of NK cell activity using other CFS defining criteria (i.e. Canadian [21] or International Consensus Criteria [22]) were found. Tables 1 and 2 summarize these 17 studies investigating levels of NKCC in patients with CFS compared to normal controls. In Table 1 the method used to measure NKCC was the standard ^{51}Cr release assay. Table 2 reports results of studies that utilized a different methodology, flow cytometry, to quantitate NKCC. The erythroleukemia cell line, K562, which was derived from a patient with chronic myelogenous leukemia (CML) [23], was used as the target in all the studies except for one [24]. K562 cells are non-adherent, positive for the bcr:abl fusion gene, lack the MHC complex required to inhibit NK activity, and are killed by NK cells.

The first investigation of NKCC in CFS identified in the literature search using the CDC 1988 CD [18] was published by Klimas, et al. [7]. This research team reported a 64% decrease in median NKCC in CFS (9%) vs. normal controls (25%) ($p<0.001$). When the data were expressed in LU (defined as the number of CD56 positive cells $\times 10^3$ needed to give 30% lysis), 289% more CD56$^+$ cells were required in the CFS patients (148) vs. normal controls (38) ($p<0.001$). Wemm, et al. studied a small number of CFS patients using the CDC 1988 CD and found a significant decrease in NK cell activity ($p<0.03$) compared to age/sex matched controls [25]. Six E:T cell ratios were utilized, but no actual NKCC values were provided. Masuda, et al. compared NKCC of CFS patients vs. normal non-fatigued controls and non-CFS, fatigued controls [26]. Blood was collected between 10 AM and noon from all participants to control for circadian periodicity. NK cell activity in the CFS cohort was 72-75% lower than that of the non-fatigued controls and 53-58% lower than the fatigued controls. Over the course of several years 3 of the fatigued-non-CFS patients subsequently developed CFS. Ojo-Amaize et al. also showed a reduction in NKCC in CFS patients (n=20) vs. normal controls (n=50) ($p<0.05$). When the CFS patients were categorized based on severity of their clinical condition, an association between decreased NK cell activity and severity of CFS was discovered [27]. Barker, et al. found a 68% reduction in NKCC in 16 CFS patients compared to 12 healthy individuals [28]. In contrast, 7 patients recovering from CFS exhibited no difference in NK cell activities vs. the healthy controls. Rasmussen, et al. studied 21 CFS patients using the CDC 1988 CD and 21 age/sex matched controls [29]. No NKCC values or p-values were presented, but an analysis comparing NKCC data from the CFS patients vs. controls using a non-parametric, Mann-Whitney test was reported to be non-significant. An analysis using a parametric test, which is usually more sensitive at detecting differences was not reported. Maher, et al. [11] noted that the Rasmussen NKCC data were expressed as a percentage of the control value within each assay using a "nonconventional" data analysis approach which likely increased variability. Mawle, et al. studied 26 CFS patients and 50 age, race, and gender matched controls and found no significant difference in NKCC between the two groups, as well as between several subsets based on duration of illness, sudden or gradual onset, or health status at time of the assay [30]. However, patients with disease duration >10 years were excluded from this study, and this may have excluded many CFS

patients with depressed NKCC (see results of Fletcher et al. [12] later in this section).

See, et al. studied the in vitro effects of Echinacea and ginseng on NKCC in healthy controls and patients with CFS using K562 as targets as discussed later [31]. Without herbal exposure, NKCC was 63% lower in the CFS patients (n=20) defined using the CDC 1988 CD compared to the normal controls (n=20). See, et al. studied NKCC in a subset of CFS patients with lower expression of glycoprotein surface markers vs. normal controls [24]. Of 212 CFS patients meeting the CDC 1988 CD that were screened, 43% or 91 patients had lower CD5, CD8, and CD11a glycoprotein-based surface markers and were selected for evaluation. Instead of the standard K562 target, (HHV-6) infected H9 cells were utilized. A 63% decrease in mean NK cell activity was seen in the CFS patients (n=91) versus age and sex matched controls (n=30) ($p<0.05$). Levine, et al. studied NKCC in a family with 8 members who met the CDC 1988 and 1994 CFS CDs, 12 unaffected family members (UFM), and 8 normal controls [32]. NKCC in the affected family members with CFS was 62% lower ($p=0.006$) than that of the normal controls. The NKCC results of the UFMs were 30% lower than the controls, but were not significantly different from either of the other two groups.

% Decrease in NKCC in CFS vs. Controls	CFS Case Definition	Number of Patients and Controls Studied	Comments	Reference
↓ 64% - (median) p<0.001	1988 CDC	CFS: 30 Healthy: 73	E:T ratio 1:1, K562=target	[7]
↓ NKCC (no values provided) p<0.03	1988 CDC	CFS: 5 NC:4	Age/sex matched, published as a letter, 6 E:T ratios from 100:1 to 3:1, K562=target	[25]
↓ 72%-75% (mean) p<0.01	1988 CDC	CFS: 10 Fatigue non-CFS: 24 Healthy Non-fatigued controls: 21	2 E:T ratios, 10:1 and 20:1, K562=target	[26]
↓ NKCC (no values provided) p<0.05	1988 CDC	CFS: 20 Healthy: 50	4 E:T ratios 50:1 to 6.25:1, K562=target	[27]
↓68% (LU)	1988 CDC	CFS: 16 Healthy: 12	E:T ratios from 50:1 to 6.25:1, K562=target	[28]
No↓ seen (no values provided)	1988 CDC	CFS: 21 NC: 21	Age/sex matched, E:T ratios not provided, K562=target	[29]
No↓ seen at each of 5 E:T ratios	1988 CDC	CFS: 16 Healthy: 50	5 E:T ratios from 50:1 to 3:1, K562=target	[30]
↓63% (mean LU)	1988 CDC	CFS: 20 NC: 20	4 E:T ratios from 40:1 to 5:1, K562=target	[31]
↓63% (mean LU) p<0.05	1988 CDC	CFS: 91[a] NC: 30	Target cell = (HHV-6)- infected H9 cells, 4 E:T ratios 40:1 to 5:1	[24]
↓62% (median LU) p<0.006	1988 and 1994 CDC	CFS: 8 UFM: 12 NC: 8	Family members with CFS and unaffected family members (UFM), 4 E:T ratios 50:1 to 6:1, K562=target	[32]
↓42% (mean) p<0.003	1994 CDC	CFS: 29 Healthy: 29	Series of 29 consecutive CFS patients; E:T ratio 1:1, K562=target	[9]
↓46% p<0.001	1994 CDC	CFS: 30 Healthy sedentary controls: 19	E:T ratio 1:1, K562=target	[34]
↓57% (median) p<0.001	1994 CDC	CFS: 176 Healthy: 230	E:T ratio 1:1, K562=target	[12]

NC: Normal Controls; LU: Lytic Units
[a]Subset of CFS patients meeting the CDC 1988 CD, but with lower levels of CD5, CD8, and CD11a mononuclear cell membrane receptors.

Table 1: Studies investigating NK cell cytotoxicity in CFS using ^{51}Cr release assays.

Fletcher, et al. [9] was the first study identified that studied NK cell activity in CFS patients defined using only the CDC 1994 CD [33]. Similar to CFS patients defined by the CDC 1988 CD, NKCC was also found to be reduced. NKCC was 42% lower in a series of 29 consecutive CFS patients vs. 29 healthy controls. The mean NKCC was 21% in the CFS patients vs. 36% in the controls ($p<0.003$). Maher et al. also used the CDC 1994 CD and found NKCC to be 46% lower in 30 CFS patients vs. 19 healthy sedentary controls ($p<0.001$) [34]. Fletcher, et al. studied 176 CFS patients using the CDC 1994 CD [12] with recommendations for resolving ambiguities [35] and 230 healthy donors. The CFS subjects had a duration of CFS from onset of symptoms of 2 to 25 years with an average onset of 10 years. Median (25-75th percentile) NKCC was 57% lower in CFS cases vs. healthy controls ($p<0.001$). The NKCC data were also expressed using a nonparametric receiver operating characteristics (ROC) analysis, which indicated an ability of NKCC to discriminate between CFS cases and healthy controls. Tirelli, et al. studied NKCC using an ^{111}In release assay and K562 as targets in 12 CFS patients meeting the CDC 1988

CD [8]. Six CFS patients were reported to have a decrease NK cell activity compared to normal donors. However, since no comparison of overall NKCC values between CFS patients and normal controls was reported, this study was not able to be included in Table 1.

% Decrease in NKCC in CFS vs. Controls	CFS Case Definition	Number of Patients and Controls Studied	Comments	Reference
↓ 60% p<0.05	1994 CDC	CFS: 10 Healthy: 10	E:T ratio=25:1, K562=target	[39]
↓~44%[a] p<0.05	1994 CDC	CFS: 71 Healthy: 50	E:T ratio=25:1, K562=target	[38]
↓~ 62% Baseline p<0.05 ↓~ 57% 6 months p<0.05 ↓~ 83% 12 months p<0.05	1994 CDC	CFS: 65 Healthy: 21	E:T ratio=25:1, K562=target	[40]
E:T ↓~ 16% 12.5:1 NS ↓~ 27% 25:1 p<0.05 ↓~ 37% 50:1 p<0.05	1994 CDC	CFS: 30 Healthy: 25	3 E:T ratios were studied, K562=target	[13]
NS=p>0.05 [a]~before % decrease in NKCC indicates values were obtained from data contained in Figures referenced in the text.				

Table 2: Studies investigating NK cell cytotoxicity in CFS using flow cytometry assays.

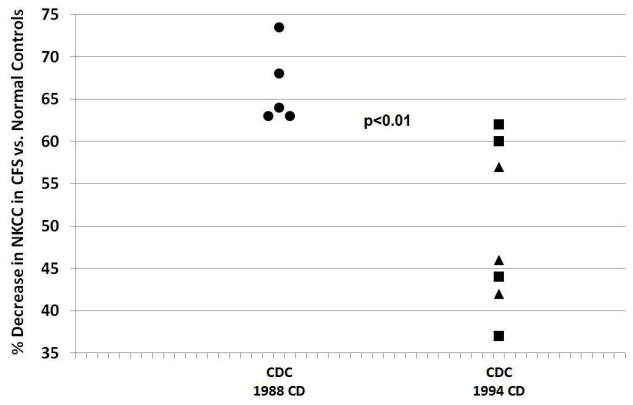

Figure 1: Comparison of percent decrease in NKCC/NK cell activity for CFS patients vs. normal controls for two populations of CFS patients defined by either the CDC 1988 or 1994 CD shows a significant difference (p<0.01). Each symbol represents an individual study from Tables 1 or 2 that reported actual values which could be presented as a percent decrease in NKCC in the first column and used either the CDC 1988 or 1994 CD (n=12), but not both CDs (n=1) as shown in the second column. The two percent decreases in NKCC values for Masuda [26], ↓75% (E:T=10:1) and ↓72% (E:T=20:1) were averaged. Two studies in Table 1 reporting a decrease in NKCC did not provide a NKCC numerical value and therefore could not be included in Figure 1 (Wemm [25]; Ojo-Amaize [27]). The initial baseline evaluation was used from the longitudinal study of Brenu [40]. For Brenu [13] data from the E:T=50:1 evaluation were used.● = 5 studies that used the CDC 1988 CD (all 5 studies used the ^{51}Cr release assay);▲= 3 studies that used the CDC 1994 CD and the ^{51}Cr release assay; ■= 4 studies that used the CDC 1994 CD and flow cytometry.

Table 2 summarizes studies evaluating NKCC in patients with CFS using flow cytometry instead of the "gold standard", ^{51}Cr release assay [36]. A comparison of NKCC evaluated by ^{51}Cr release vs. flow cytometry in 10 healthy women aged 18 to 39 years using cryopreserved peripheral blood mononuclear cells (PBMCs) found that the relationship of NKCC (LU) between the two methods was strongly positive (r=0.79, p=0.006) [37]. NKCC detected by ^{51}Cr release was higher than that seen by flow cytometry for all 10 subjects studied. The flow cytometry methodology required a larger blood sample and generated greater costs compared to the ^{51}Cr release assay. The authors concluded that there was no compelling reason to adopt flow cytometry over ^{51}Cr release for evaluation of NKCC. However, flow cytometry is an important tool to investigate potential mechanisms of reduced NKCC [34,38], which are not discussed in this review. Flow cytometry is also more easily adapted to a clinical study since ^{51}Cr is a research laboratory procedure not generally used by clinical laboratories.

Brenu et al. was the first identified study which utilized flow cytometry to measure NKCC in CFS patients vs. normal controls [39]. CFS patients were identified using the CDC 1994 CD and had a duration of CFS of more than 5 years. A 60% decrease in NKCC in the CFS cohort (n=10) compared to healthy controls (n=10) was seen (p<0.05). Brenu et al. studied NKCC in CFS patients (n=71) and non-fatigued healthy controls (n=50) 25-65 years of age [38]. Approximately 44% decrease in NKCC expressed in terms of LU was seen in the CFS cohort vs. controls (percent decreases in NKCC were obtained from Figure 2 of Brenu, et al. [38]). Brenu et al. conducted a longitudinal study of NK cell activity in 65 CFS patients and 21 non-fatigued controls at 3 time points, baseline, 6 and 12 months [40]. NKCC was consistently decreased compared to the control group at each of the three time points (p<0.05). The percent decreases in NK cell activity were obtained from the NKCC data in Figure 1(A) [40] and showed an approximately 62%, 57% and 83% decrease in CFS vs controls at baseline, 6 months, and 12 months, respectively. Brenu, et al. studied NKCC in 30 CFS patients and 25 non-fatigued controls using 3 E:T ratios [13]. The least discriminating E:T ratio was 12.5:1 which yielded~16% decrease in CFS and was not significant (p>0.05). NKCC testing using both the 25:1 and the 50:1 E:T ratio was significantly lower in CFS patients vs. controls (Table 2). Percent decreases in NKCC shown in Table 2 were obtained from data in Figure 3a [13].

Relationship between NK cell cytotoxicity and severity of CFS

Table 3 summarizes studies investigating the relationship between NK cell cytotoxicity and severity of CFS. Barker et al. found a 68% decrease in NKCC in 16 CFS patients meeting the CDC 1988 CD compared to 12 healthy controls [28]. In contrast, 7 patients either recovering or recovered from CFS did not exhibit a decrease in NKCC vs. the healthy controls. Masuda, et al. found NKCC was 72-75% lower in 10 CFS patients than in 21 healthy non-fatigued controls ($p<0.01$) and 53-58% lower than in 24 chronically fatigued patients ($p<0.05$), who did not meet the CDC 1994 CD [26]. Three of these chronically fatigued patients subsequently progressed to CFS over several years of follow up.

Ojo-Amaize, et al. studied NKCC in 3 cohorts of patients with increasing severity of CFS defined using the CDC 1988 CD [27]. The NK cell activity of the 3 combined groups (n=20) was significantly lower than that of a healthy control group (n=50) ($p<0.05$). The number of patients in each severity group and corresponding mean NKCC values are shown in Table 3. Of the 3 severity groups, only the middle (more severe) cohort had NKCC that was significantly lower than that of the control group ($p<0.05$), but the pattern of decline in NK cell activity with increasing severity of CFS supports a relationship between NKCC and CFS severity. Of interest, the least severe cohort consisted of patients with the shortest duration of illness and NK cell activity was not significantly different from the healthy controls.

Diaz-Mitoma, et al. randomly assigned CFS patients meeting the CDC 1988 and 1994 CDs to a 12 week single blinded study of Isoprinosine® versus placebo [41]. Based on the patient's self-reports to the Investigator, clinical improvement was seen in 6 of the 10 patients treated for 12 weeks with Isoprinosine. There was a significant difference in NKCC at 12 weeks in the 6 clinically improved CFS patients versus the 4 non-improved patients (23.7 vs. 14.7 LU, respectively ($p<0.03$). In addition, the increased NK cell activity observed in the clinically improved patients correlated with the duration of Isoprinosine treatment ($p<0.03$).

Results Support a Relationship Between a Lower NKCC and Greater CFS Severity	CFS Case Definition	Number of Patients and Controls Studied	Comments	Reference
Yes	1988 CDC	CFS: 16 Recovering from CFS: 7 Healthy: 12	NKCC of CFS pts was ↓ 68% compared to healthy control. In contrast, 7 patients either recovering or recovered from CFS did not exhibit a decrease in NKCC vs. the healthy controls. ^{51}Cr-release assay, K562=target	[28]
Yes	1988 CDC	CFS: 10 Fatigued, non-CFS: 24 Healthy: 21 (non-fatigued controls)	2 E:T ratios 10:1 and 20:1, ^{51}Cr-release assay, K562= target	[26]
Yes	1988 CDC	Least severe CFS (n=10), more severe CFS (n=7), most severe CFS (n=3) Healthy: 50	Mean NKCC= 61 LU (Least severe); 18 LU (more severe); 8 LU (most severe), ^{51}Cr-release assay, K562= target	[27]
Yes	1988 and 1994 CDC	Isoprinosine treated CFS: 10 Placebo treated CFS: 6	Improvement in NKCC seen in 6 clinically improved CFS pts vs. 4 non-improved pts ($p<0.03$). ^{51}Cr-release assay, K562= target	[41]
Yes	1994 CDC	CFS: 41 female patients classified into 2 groups Low NKCC, n=22 Normal NKCC, n=19	CFS cohort with low NKCC had less vigor, more daytime dysfunction, and more cognitive impairment compared to the CFS cohort with normal NKCC. ^{51}Cr- release assay, K562= target	[42]
Yes	1994 CDC	Severe CFS: 18 Moderate CFS: 23 Healthy: 22	3 E:T ratios 12.5:1, 25:1, 50:1 were studied using flow cytometry, K562=target	[43]
LU: Lytic Units				

Table 3: Studies investigating relationship between NK cell cytotoxicity and severity of CFS.

Siegel et al. studied the relationship between NKCC and CFS severity by classifying 41 female CFS patients meeting the CDC 1994 CD into a low NKCC cohort (n=22) and a normal NKCC cohort (n=19) [42]. These two subgroups were then compared on cognitive functioning, assessments of fatigue and vigor, and daytime dysfunction. Compared to CFS patients with normal NK cell activity, patients with reduced NK cell activity reported less vigor more cognitive difficulty and poorer daily functioning. Hardcastle, et al. studied the relationship between immune dysfunction including NKCC and symptom severity in patients with CFS using the CDC 1994 CD [43]. Two groups of CFS patients including moderate/mobile (n=23) and severe/bedridden (n=18) were compared with a healthy non-fatigued control group (n=22). There was no significant difference in mean age or gender between the 3 groups. In all 3 E:T ratios (12.5:1, 25:1, and 50:1) studied, there was a significant decrease in NKCC in moderate ($p<0.017$) and severe CFS patients ($p<0.001$) compared to the healthy controls. NK cell activity was also more reduced in the severe CFS cohort compared to the moderate severity group for all 3 ratios, but the difference was not statistically significant.

Therapeutic approaches for improvement of NK cell cytotoxicity in CFS

Table 4 summarizes studies evaluating the ability of various agents to augment NK cell activity. See et al. treated 30 CFS patients with rIFN-α-2a (3 million units 3 times/week SQ) or placebo for 12 weeks in a double-blind crossover study and evaluated NK cell activity and other immune parameters at baseline and at 12 weeks [44]. Although mean NKCC increased 47.3% ($p<0.05$) following 12 weeks of IFN-α therapy, there was no significant change in any other immune parameters or in QOL scores. The failure to see improvement in QOL scores overall may be related to the various toxicities seen with the IFN-α treatment as summarized in footnote (a) of Table 4. However, the subset of CFS patients with low NK function and normal lymphocyte proliferation at baseline (n=7) demonstrated significant increases in both mean NKCC ($p<0.01$) and in QOL ($p<0.05$) compared to baseline. See et al. studied augmentation of NK cell activity in 20 CFS patients and 20 healthy controls using a ^{51}Cr release assay and K562 as targets [31]. Extracts of two herbs, Echinacea purpurea and Panax ginseng, were added separately at 6 concentrations to ^{51}Cr labeled K562 targets and PBMCs using 4 E:T ratios and incubated for 4 hours. Both Echinacea and ginseng at concentrations of ≥ 0.1 to 100 μg/ml increased NKCC in both CFS patients and normal controls. The maximum increase for Echinacea occurred at concentrations of 100 μg/ml and 10 μg/ml in CFS patients (~147%) and normal controls (~69%), respectively. Similarly, the maximum increase for ginseng was at 100 μg/ml for both CFS patients (~87%) and normal controls (~42%). NKCC data was obtained from Figure 1 [31].

% Change in NKCC [Treatment]	CFS Case Definition	Number of Patients and Controls Studied	Comments	Reference
↑ 47.3 % (mean) [rIFN-α-2a] in vivo[a]	1988 CDC	CFS: 30 patients started IFN-α, 4 pts discontinued[a], 26 were evaluated for NKCC	3 E:T ratios 20:1, 10:1, and 5:1 used in a ^{51}Cr release assay, K562=target	[44]
↑ 87% (mean) [extracts of *Panax ginseng*] in vitro	1988 CDC	CFS: 20 NC: 20	^{51}Cr release assay, K562=target	[31]
↑ 147% (mean) [extracts of *Echinacea purpurea*] in vitro	1988 CDC	CFS: 20 NC: 20	^{51}Cr release assay, K562=target	[31]
↓ 22.3% CFS (mean) ↑ 45.8% NC (mean) [L-arginine] in vitro	1988 CDC	CFS: 20 NC: 21	LDH[b] release assay instead of ^{51}Cr, K562=target	[45]
↑ ~ 114% (mean) [Glyconutrients] in vitro	1988 CDC	CFS: 91[c] NC: 30	Target cell= (HHV-6)-infected H9 cells, 4 E:T ratios 40:1 to 5:1 used in a ^{51}Cr release assay	[24]
↑ 223% (median) in 6 of 10 clinically improved patients [Isoprinosine] in vivo	1988 and 1994 CDC	Isoprinosine treated CFS: 10 Placebo treated CFS: 6	↑ 33% (median) in 4 non-clinically improved CFS pts, ^{51}Cr release assay, K562=target	[41]
↑ 178% (mean) ↑ 100% (median) [rintatolimod] in vitro	1988 and 1994 CDC	CFS: 15	E:T ratio 12.5:1, flow cytometry assay, K562=target, mean age = 47.5 and median age = 46.0 years, 67% were female	Hemispherx Unpublished Data

NC = Normal Controls

[a]3 million units of rIFN- α-2a was given 3 times/week SQ. Four CFS patients discontinued early because of IFN toxicity: neutropenia (2), palpitations (1), worsened fatigue (1). In addition, 15 patients reported significant flu-like symptoms (4), new onset diarrhea (2), and hair loss (9). NKCC data was obtained from Figure IA.

[b]Lactate dehydrogenase

[c]Subset of CFS patients meeting the CDC 1988 CD, but with lower levels of CD5, CD8, and CD11a mononuclear cell membrane receptors.

Table 4: Studies investigating therapeutic approaches for augmentation of NK cell cytotoxicity in CFS.

Ogawa et al. showed that L-Arginine activated NK cell activity in 21 healthy controls, but did not increase NK cell activity in 20 CFS patients meeting the CDC 1988 CD [45]. A 24 hour in vitro exposure of PBMCs to 30 mmol L-arginine/liter increased NKCC 45.8% from 10.7% to 15.6% in healthy controls compared to a decrease of 22.3% in CFS patients from 9.4% to 7.3%. The authors concluded that a possible dysfunction in NO-mediated NK cell activation may exist in CFS patients [45]. See, et al. also studied the effects of glyconutrients on NK cell activity in patients with CFS vs. normal controls [24]. Of 212 CFS patients meeting the CDC 1988 CD that were screened, 43% or 91 patients had lower CD5, CD8, and CD11 a glycoprotein- based surface markers and were selected for evaluation. Instead of the standard K562 target, H9 cells infected with HHV-6 were utilized. As shown in Table 1, a 63% decrease in mean NKCC was seen in this subset of CFS patients (n=91) versus age and sex matched controls (n=30) without addition of glyconutrients ($p<0.05$). Addition of a glyconutrient preparation to the target and effector cells with incubation for 8 hours enhanced the NKCC in patients with CFS compared to controls with no glyconutrients ($p<0.01$) (Table 4). Diaz-Mitoma, et al. randomly assigned CFS patients to receive inosine pranobex (Isoprinosine®) (n=10) or a methylcellulose placebo (n=6) [41]. NKCC was measured at Week 0 prior to treatment and after 12 weeks of randomized

treatment. Six of the 10 CFS patients receiving Isoprinosine® for 12 weeks showed clinical improvement based on the patients' self-reports to the investigator and the results of an Investigator Assessment Form evaluating symptoms during each visit. The clinical assessments were carried out independently from the NKCC evaluation. Median placebo-adjusted NKCC increased 223% in the subset of 6 CFS patients showing clinical improvement compared to a 33% increase in the 4 CFS patients without clinical improvement (p=0.03). In vitro exposure of PBMCs from CFS patients, fulfilling both the CDC 1988 [18] and 1994 [33] CDs, to rintatolimod increased NKCC 100-178%. Separately, in vivo treatment of severe cases of CFS, also fulfilling both CDC CDs, with rintatolimod in a Phase III randomized, placebo-controlled trial significantly increase exercise tolerance compared to placebo and was generally well-tolerated [46].

Discussion

Three of 4 studies of NK cell activity in patients with chronic fatigue conducted prior to 1988 [15,16,19] did not use a recognized case definition of CFS, but nonetheless reported a decrease in NK cell activity compared to healthy controls. One of the 4 studies [20] did not report a difference in NKCC from controls, and a retrospective analysis showed that 20 of the 26 patients studied did not fulfill the CDC 1988 CD for CFS. Since the publication of the CDC 1988 and 1994 CDs, 17 studies were published that utilized one or both of these criteria and compared NKCC in CFS patients vs. healthy controls (Tables 1 and 2). Eighty-eight percent of these publications (15/17) concluded that NK cell activity was decreased in CFS patients compared to normal controls.

Two studies [29,30] did not report lower NK cell activity in CFS. Rasmussen studied 21 CFS patients using the CDC 1988 CD and 21 age and sex-matched normal controls. However, a nonconventional method of analysis of the NK data was utilized [11]. The other study which did not detect a difference in NK cell activity between CFS patients and healthy controls also used the CDC 1988 CD [30]. NKCC in 16 CFS patients was compared to 50 age, sex, and race-matched healthy controls. Five E:T ratios from 50:1 to 3:1 were studied and the NKCC results were consistent across each E:T ratio. Although, the CDC 1988 CD was utilized, only a subset of CFS patients meeting the CDC 1988 CD were studied. CFS patients who had been sick > 10 years were not included in the study. Since the duration of illness in CFS commonly exceeds 10 years [12], a large proportion of CFS patients who may have been most likely to exhibit low NK cell activity [27] were excluded. Fletcher, et al. studied 176 CFS patients meeting the CDC 1994 CD and found a range of 2 to 25 years from onset of symptoms with an average of 10 years [12]. The mean duration of CFS symptoms in a cohort of 234 severely ill patients with CFS was also 10 years [46]. Therefore, the study conducted by Mawle et al. [30], which found no significant difference in NKCC between CFS patients and normal controls, may have excluded many CFS patients who were more likely to have been symptomatic and had a lower NK cell activity [27].

The vast majority (88%) of published studies evaluating NK cell activity in patients meeting the CDC 1988 or 1994 CD concluded that CFS is associated with a reduction in NKCC compared to healthy controls. Both of the studies [29, 30] that did not find a difference in NKCC between CFS patients and normal controls contained potential flaws which may have influenced the results. Decreased NK cell activity in CFS was found using both the ^{51}Cr release assay (Table 1), as well as, flow cytometry (Table 2). Of the 15 studies summarized in Tables 1 and 2, reporting a decrease in NKCC in CFS, 7 utilized the CDC 1988 CD, and 7 reported using the CDC 1994 CD. One study referenced using both criteria [32]. Compared to the CDC 1988 CD, the CDC 1994 CD requires a fewer number of symptoms of CFS [18, 33]. Figure 1 shows the percent decrease in NKCC for the 5 studies in Table 1 using the CDC 1988 CD that reported a percent decrease NKCC value compared to the 7 studies in Tables 1 and 2 that utilized the CDC 1994 CD. The single study reporting the use of both the CDC 1988 and 1994 CDs was not included in the analysis. As shown in Figure 1, there is no overlap in the ranges of the percent decreases in NKCC values for the studies using the CDC 1988 CD (63-73.5%) compared to the CDC 1994 CD (37-62%). The mean percent decrease in NKCC for the CFS populations defined using the CDC 1988 CD (66.3%) was significantly greater than that for the CFS patients defined using the CDC 1994 CD (49.7%) ($p<0.01$, using both the T-test and Wilcoxon-Mann-Whitney test). There was no significant difference in the percent decrease in NKCC detected for the CFS population defined using the CDC 1994 CD comparing the ^{51}Cr release assay (48.3%) vs. flow cytometry (50.7%) ($p>0.5$, for both the T-test and Wilcoxon-Mann-Whitney test). The data in Figure 1 are consistent with the findings of the 6 publications in Table 3 showing a greater decrease in NKCC with increase in CFS symptom severity based on the lower symptom requirement for the CDC 1994 CD [33] compared to the CDC 1988 CD [18]. The initial 1988 CD required new onset of a debilitating fatigue, that reduced daily activity levels greater than 50% for 6 months or longer. In addition, to meet the 1988 CD patients must fulfill a symptom/physical criteria which included at least 6 of the specified 11 symptoms plus 2 of the specified 3 physical criteria or 8 of the symptoms. The 1994 CD required an unexplained chronic fatigue of 6 months duration that resulted in a substantial reduction in previous activities plus only 4 or more of 7 specified symptom criteria. No physical criteria were specified. Based on the data in Tables 1 and 2 and Figure 1, the decrease in NK cell activity seen in CFS patients compared to healthy controls has been reproduced repeatedly, not only using the ^{51}Cr release assay (Table 1), but also using flow cytometry (Table 2).

NKCC has been reported to have potential as a biomarker for CFS [12]. The percent decrease in NKCC in CFS patients compared to normal controls ranged from 42% to 75% using the ^{51}Cr-release assay (Table 1). This decrease in NKCC was obtained using cohorts of CFS patients and normal controls with median sizes of 29 and 21 individuals, respectively. How useful is NKCC as a biomarker for CFS in an individual CFS patient? Fletcher, et al. studied NKCC in 176 CFS patients and 230 healthy donors and expressed the data using a ROC plot [12]. This analysis showed an ability of NKCC to discriminate between CFS and healthy controls. However, the discrimination was not sufficient to use NKCC alone as a diagnostic biomarker. For example, the data in Figure 1 of reference 12 show that to obtain 80% sensitivity (e.g., to be able to detect 80% of the true CFS positive patients), the specificity would be about 61-62% (e.g., only 61-62% of the population selected would actually have CFS). In order to obtain 90% sensitivity, the specificity decreases to 40-41%. However, as discussed below, NKCC might have greater success as a biomarker for CFS patients with greater disease severity.

Table 3 summarizes 6 publications containing NKCC data from CFS patients showing a relationship between the severity of CFS and NK cell activity. All 6 studies present data which supports a relationship between a lower NK cell activity and a higher level of CFS symptom severity. Three studies identified CFS patients using the CDC 1988 CD, two studies used the CDC 1994 CD, and one utilized

both. In total, 145 patients with CFS were studied. Five studies employed the ^{51}Cr release assay, while one used flow cytometry. No publications were found that studied NK cell activity as a function of CFS symptom severity that reached a different conclusion. Thus, these data support NKCC as a potential biomarker for symptom severity in patients with CFS. Confirmation would require additional laboratory and clinical research.

Low NKCC in patients with CFS represents a potential therapeutic target. Table 4 summarizes 6 studies that investigated the ability of seven different agents to augment NKCC in patients with CFS. Four studies investigated augmentation of NKCC using PBMCs from CFS patients using in vitro exposure methodology, so direct clinical correlates were not possible. Four of the 5 agents studied showed in vitro augmentation of NKCC in CFS samples following treatment with ginseng, Echinacea, glyconutrients, and rintatolimod, while one study [45] reported a decrease in NKCC with L-arginine. This study was unique in that it utilized a LDH release assay instead of the standard ^{51}Cr release assay, but did report an increase in NKCC with L-arginine in normal adults. All of the other studies utilized the ^{51}Cr release assay except for the rintatolimod (Ampligen®) study which used flow cytometry. However, a prior study had shown an ability of rintatolimod (Poly I : Poly $C_{12}U$) to increase NKCC in humans (in vitro) using the ^{51}Cr release assay [47]. Two studies summarized in Table 4 examined NK cell activity prior to and following randomized treatment of CFS patients with IFN-α-2a vs. placebo or Isoprinosine vs. placebo, respectively [44,41]. Both studies reported increases in NK cell activity with the drug treatments. Increases in NKCC and clinical improvement (QOL scores) were seen in only the subset of patients treated with IFN-α-2a who had a decrease in NKCC and normal lymphocyte proliferation at baseline [44]. Diaz-Mitoma, et al. [41] found that NKCC increased 223% in the cohort of CFS patients showing clinical improvement to 12 weeks of Isoprinosine treatment compared to the patients without clinical improvement. The duration of treatment also correlated with the increases in NK cell activity ($p<0.03$). These results are consistent with the findings in Table 3 and Figure 1 showing a relationship between increased disease severity and lower NK cell activity.

An important future research direction in CFS is to develop safe and effective therapy for this severely debilitated and chronically ill patient population. Low NK cell activity is commonly seen in CFS and is associated with increased symptom severity. Thus, low NKCC in CFS provides an important clue to the pathophysiology of the illness. Approaches to safety augment NKCC in this subset of CFS patients should be explored. Determination of whether correction or augmentation of the decreased NKCC seen in CFS by treatment intervention will lead to clinical improvement of CFS symptoms and other clinical outcomes is an important clinical question that will require well-designed, prospective clinical trials that control for variables that influence NK cell activity [48].

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