Autoimmun Highlights (2013) 4:69–80
DOI 10.1007/s13317-013-0051-x

REVIEW

Natural killer cells in patients with severe chronic fatigue syndrome

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Received: 28 November 2012 / Accepted: 6 March 2013 / Published online: 16 April 2013

Abstract Maintenance of health and physiological homeostasis is a synergistic process involving tight regulation of proteins, transcription factors and other molecular processes. The immune system consists of innate and adaptive immune cells that are required to sustain immunity. The presence of pathogens and tumour cells activates innate immune cells, in particular Natural Killer (NK) cells. Stochastic expression of NK receptors activates either inhibitory or activating signals and results in cytokine production and activation of pathways that result in apoptosis of target cells. Thus, NK cells are a necessary component of the immunological process and aberrations in their functional processes, including equivocal levels of NK cells and cytotoxic activity pre-empts recurrent viral infections, autoimmune diseases and altered inflammatory responses. NK cells are implicated in a number of diseases including chronic fatigue syndrome (CFS). The purpose of this review is to highlight the different profiles of NK cells reported in CFS patients and to determine the extent of NK immune dysfunction in subtypes of CFS patients based on severity in symptoms.

Keywords Chronic fatigue syndrome · Natural killer cells · Cytotoxicity · Perforin · Granzymes

Electronic supplementary material The online version of this article (doi:10.1007/s13317-013-0051-x) contains supplementary material, which is available to authorized users.

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Introduction

Natural killer (NK) cells are granular lymphocytes originating from the CD34 hematopoietic progenitor cell lineage and are found in the peripheral blood, spleen, bone marrow and lymph nodes. The composition of NK cells in comparison to the total lymphocyte population is about 15 % [1]. These cells are important in the principal innate immune defence in response to pathogen invasion following recognition. NK cells are imperative during viral and microbial infection and tumour development, aiding in the body's immunity through cytokine secretion and cytotoxic activity, which induces apoptosis in target cells. Thus, NK cells are vital for pathogen clearance prior to the adaptive immune response. Aberrant production of cytokines and induction of cytotoxic activity are related to a number of disease presentations such as rheumatoid arthritis [2], chronic obstructive pulmonary disorder [3], neurological conditions including Alzheimer's and multiple sclerosis [4, 5] and cancers [6–9]. In particular, NK cell cytotoxic dysfunction has been associated with chronic fatigue syndrome (CFS).

Subsets of NK cells

Natural killer cells can be grouped into two types, however, further classifications generates at least five subsets of NK cells [10, 11]. These cells are mainly grouped according to the presence of the Fc gamma receptor III (CD16) and the neural cell adhesion molecule (CD56). The density of these markers on the NK cells defines the classification of different subsets of NK cells. Thus, NK cells can be grouped into $CD56^{bright}CD16^{dim}$, $CD56^{bright}CD16^{-}$, $CD56^{-}CD16^{bright}$, $CD56^{dim}CD16^{bright}$ and $CD56^{dim}CD16^{-}$NK cells [10, 11]. About 90 % of the NK cells in the periphery are $CD56^{dim}CD16^{bright}$ while 10 % express $CD56^{bright}$ on their cell surfaces [10]. NK cells with a high density of the CD16 molecules are considered highly cytotoxic and secrete low levels of cytokines while those densely populated with CD56 markers are the dominant producers of NK-related cytokines and are less cytotoxic [10]. The cytokines secreted by these NK cells include granulocyte macrophage colony-stimulating factor (GMC-SF), IFN-γ, TNF-α, IL-10 and IL-13. The ability to induce cytotoxic activity and produce cytokines is vital for sustained physiological homeostasis.

NK cell receptors

Natural killer cells express a number of activating and inhibitory receptors generated from genes with variable or conserved sequences. The most variable and polymorphic NK receptors are the killer cell immunoglobin-like receptor (KIR) family of receptors while the others such as NKG2D are highly conserved [12]. KIRs are a family of inhibitory receptors that reside as transmembrane proteins, with two or more extracytoplasmic immunoglobin-like domains with either short or long cytoplasmic tails that have an immunoreceptor tyrosine-based activation or inhibition motif (ITAM and ITIM, respectively). KIRs contain a long cytoplasmic tail and do not associate with adaptor molecules. The activation of KIRs is dependent upon the recognition of MHC class I molecules. The primary function of these receptors is the inhibition of cytotoxic activity—the inhibitory KIRs are associated with having a longer cytoplasmic tail. However, some KIRs containing ITAM motif, KIR2DS/3DS, are known to stimulate cytotoxic activity [13].

Natural killer cells undergo cytotoxic activation by exogenous activation of surface receptors. Activating NK receptors including, NKp46, NKp30 and NKp44, all share the association of a signalling peptide existing on cytoplasmic tail, known as ITAM. ITAMs are highly conserved peptides containing tyrosine residues [14]. The ITAM peptide is a crucial intermediate between activation of the surface receptor and downstream effector signalling, which in the case of NK cell is cytotoxic activation and the production of cytokines including IFN-γ, tumour necrosis factor β (TNF-β), IL-10 and IL-13. The activation receptors of NK cells have a broad specificity and can be activated by target cell antibody interaction, such as that of an antigen presenting cell or B cell, or through the recognition of the MHC class I-like complex. It has been indicated that numerous receptor activation and cross linking between these receptors is required for NK cytotoxic activation [15].

Natural killer cell activation predominantly relies on receptors. Upon recognition of the adaptor molecule, the ITAM components of the receptor are activated through the phosphorylation of its tyrosine residues [14]. This temporary binding site now has an affinity to activate of ITAM, which leads to the recruitment of a src-family kinase (SH2) pathway and downstream syk/ZAP70 transcription. Syk acts through the PI3K-dependent pathway to activate Rac1, PAK1, MEK and ERK pathways increasing calcium entry, degranulation, recruitment of perforin and granzyme contained in lytic granules and cytokine gene transcription [16, 17]. These activating receptor molecules on target cells may be antibodies or MHC class 1-like molecules, requiring activation of multiple receptors and receptor cross-linking to activate cytotoxicity [18, 19]. Inhibition of NK cells occurs in the absence of a structurally sufficient MHC class I molecule, giving rise to the 'missing self' theory of cytotoxic inhibition [20]. Recognition of the target cell MHC class I, prompts ITIM phosphorylation at the tyrosine residues to recruit and activate SHP-1/2 phosphatases. SHP1/2 dephosphorylates activated ITAM pathway constituents, Syk and ZAP70, thereby inhibiting cytotoxic activation and cytokine production.

NK cell cytotoxic activity

Natural killer cells can induce apoptosis in target cells through granule-mediated and non-granule-mediated pathways. Granule-dependent cytotoxic induction is the most specialised cytotoxic function of the NK cell [21, 22]. However, the significance of non-granule-mediated pathways is evident in the diversity of lethality of the NK cell including antibody-dependent cell-mediated cytotoxicity [23–27], TRAIL and FasL death receptor pathways [28–30].

The relevance of the cytolytic granule-mediated pathway to the CFS disease state is supported by a growing body of evidence highlighting cytotoxic dysfunction and the immune system [31–37], summarised by Bansal et al. [38]. By activation of the granule-mediated pathway, NK cells secrete perforin and granzymes into the target cells. Perforin is a protein that either forms pores on the plasma

membrane of the target cells to facilitate the passage of granzymes into the target cells or fragments the host cell endosomes, which contain granzymes [21]. They may also be directly involved in cytolytic activity and once in the target cells, granzymes bind to cell organelles to activate either caspase-dependent or -independent apoptosis. These mechanisms include nuclear envelope disruption leading to DNA degradation, disruption of mitochondrial transmembrane potential and independently activates Ape1-mediated bcl-2 overexpression [39]. NK cells may also contain a memory component that assists in future invasions by the same antigen. The primary pro-apoptotic component of NK cells—granzymes, are currently categorised into five types, only three of which have directly known functions—granzymes A, B and K [40].

Granzymes A and K have similar functions and are known to activate slow apoptosis while granzyme B is associated with the activation of rapid apoptosis [41]. Granzyme B induces a caspase-dependent mechanism of apoptosis while granzyme A is caspase independent, inducing cell death through single stranded DNA degradation, disrupting plasma membrane integrity and mitochondrial transmembrane potential [39, 42–44]. The exact mechanism of perforin is still under investigation, however, mounting evidence tends to support oligomerisation within the target cell membrane, leading to the formation pore-like structures [45, 46]. These pores are then the gateway for NK cell-derived granzymes to enter the target cell and elicit their effect. NK cell cytotoxic activity also occurs through a number of other secondary pathways including IFN-γ, TNF-α and Fas-ligand pathways, the dysregulation of which may be involved in the mechanism of CFS.

Chronic fatigue syndrome

Presently, an indefinable aetiology and mechanism of CFS precludes effective diagnosis posing substantial anxiety among patients and family members. CFS is a heterogeneous disorder with multi-factorial characteristics affecting physiological processes including, endocrine, neurological, immunological and metabolic processes [47–54]. Substantial physical and mental weaknesses are associated with CFS including but not limited to severe disabling fatigue, interruptions in sleep, headaches, swollen lymph nodes, cognitive disturbances and muscle pain in the absence of swelling [55]. CFS is neither age- nor gender-specific, however, females are more likely to be affected than males [56–58]. CFS is an unexplained disorder with a prevalence of 0.4–1 % worldwide [59]. Nonetheless prevalence of CFS varies among patients [60].

NK cells in CFS

Chronic fatigue syndrome is known to be associated with a reduction in NK lytic activity and in some cases an irregular distribution in the levels of NK subtypes. In a range of studies, NK cytotoxic activity has been measurably decreased as compared to healthy controls [31, 33, 34, 61–64]. There is no standard definition for CFS, however, a number of criteria have been generated to assist physicians in disaffecting CFS from other known and characterised disorders [59]. Similarly, our longitudinal investigations of CFS have shown that reduced cytotoxic activity in patients with CFS is maintained during the course of the disease and does not notably fluctuate or associate with seasonal changes. Incidentally, we have recently studied a group of severely bed-ridden patients, due to the symptoms of CFS. In these individuals, we have shown that, similar to the CFS patients who have some level of mobility outside the home, these patients demonstrated a significant decrease in cytotoxic activity in their NK cells. However, in previous investigations, reductions in NK cell function have been associated with reduced levels of NK $CD56^{bright}$ cells [34]. A similar trend was not observed in our severe bed-ridden population and a study of the longitudinal expression of these subsets demonstrated that these cells are not consistently reduced over time or during the course of the disease [31]. This is consistent with the NK cells subset studies in the literature where consistencies in the levels of NK cells have not been observed across all studies. The heterogeneity of CFS may be associated with these findings; however, it posits that levels of NK cells may not be an appropriate marker for identifying and distinguishing CFS from the general population. The observation of reduced NK cell cytotoxicity in both mobile and bed-ridden cases of CFS is important to the current knowledge of the disease. In the severe cases of CFS, differences in the KIR receptors may be associated with the disease presentation. Notably the transcriptional levels of some KIRs are significantly decreased in the CFS patients compared to the controls while the expression of KIR2DS5 is not observed in all CFS patients [65].

Allotypic and haplotypic differences in the expression of these KIRS may affect the induction of cytotoxic activity [66, 67]. Due to the high polymorphic nature of KIR, it is proposed that specific polymorphisms may be associated with the differences in expression of CFS patients [68]—either due to a pre-transcriptional or a compensatory means. However, studies are yet to provide details on such genomic data of KIRs in CFS patients, which may be further complicated by the heterogeneity and ambiguity of the disease presentation and symptoms. In the absence of appropriate diagnostic tools and a well-characterised suite of biomarkers, CFS remains complex. A unifying theme in

the literature is the presence of dysfunctional immune cells specifically NK cells. Most immunological studies in CFS are concerned with the cytotoxic activity and phenotypic distribution of these cells.

Equivocal levels of NK cells and phenotypes in CFS

Chronic fatigue syndrome studies associated with peripheral NK cell phenotypes or subsets are contradictory. Studies have reported increases, decreases and no change in peripheral distribution of NK cell phenotypes and overall NK cells in comparison to non-fatigued participants. Regardless of these inconsistencies, alterations in NK phenotypes have adverse consequences on immune function owing to the cytokine secretion and cytolytic properties of these cells. Characterisation of NK cells into $CD56^{bright}$ and $CD56^{dim}$ permits the determination of the distribution of cytokine producing and cytotoxic NK cells.

Overall NK cell numbers may be increased or decreased in some CFS patients [69, 70]. Similarly, $CD56^{bright}$ NK cells may be increased or decreased in some CFS patients [34, 70]. The consequence of equivocal levels of $CD56^{bright}$ NK cells in CFS patients is unknown. Nonetheless, $CD56^{bright}$ NK cells are highly resistant to apoptosis and therefore have in increased life span in comparison to the $CD56^{dim}$ NK cells [71, 72]. $CD56^{bright}$ NK cells in close relation to T cells may be increased following elevations in their numbers perpetuating autoimmune responses [73]. The presence of heightened levels of $CD56^{bright}$ NK cells may suggest the presence of inflammation in the periphery. The diversity in chemokine receptor expression on the subtypes of NK cells is related to their sites of manifestation during inflammation, hence, $CD56^{bright}$ NK cells expressing CCR5 are present in inflamed areas with high incidence of RANTES and MIP-1α and β [74, 75]. These NK cells are substantially activated following from their interactions with monocytes [76]. Interestingly, reduction in the levels of $CD56^{bright}$ NK cells is suggestive of differentiation of the $CD56^{bright}$ NK cells into the $CD56^{dim}$ NK cells [77]. Concurrent expression of high levels of $CD56^{dim}$ NK cells and low level of $CD56^{bright}$ NK cells may explain this phenomenon in CFS patients [70]. In diseases like AIDS, reduction in the levels of $CD56^{dim}$ NK cells correlates with decreases in NK cytotoxic activity [78]. The equivocal levels of NK phenotypes limits the acceptance of this assumption in CFS patients nonetheless, it is possible to posit that in some CFS patients with marked reduction in NK phenotypes and activity a similar disease profile may be observed. The heterogeneity of CFS may confuse these findings hence, levels of NK cells may not be appropriate markers for identifying and distinguishing CFS from the general population.

NK cytotoxic activity in CFS

Although NK cell phenotypes and overall NK cell numbers in the periphery are unpredictable, consistent decreases in the cytotoxic activity occur in most CFS patients [1, 5, 36, 64, 69, 79–83]. A rationale for decreases in cytotoxic activity remains to be determined, however, these may be associated with altered lytic proteins in particular perforin and granzymes [37]. Lytic proteins are important factors in the granule-dependent pathway of cytolysis. Perforin contains a membrane attack complex and a C2 domain that contains Ca^{2+} [84]. In CFS patients, perforin gene expression may be increased in conjunction with relatively low to normal levels of granzymes [32, 37, 85].

Perforin is an important indicator to cytotoxic activity as it is an absolute necessity for granule-related apoptosis [21, 86]. Incidentally, mice lacking perforin demonstrate reduced apoptosis [86, 87]. Trafficking or exportation of granzymes into the target cell is dependent on the availability of perforin thus its deficiency pre-empts decreased cytotoxic activity owing to the paucity in the available granzymes to induce apoptosis [21, 45]. Granzyme distribution in some CFS patients may be reduced [32]. It is known that during development the level of perforin in the NK cell is related to the expression of CD56. Following maturation, a substantial proportion of NK cell-related perforin is detected in the $CD56^{dim}$ NK cells in comparison to the $CD56^{bright}$ NK cells [88]. Upregulation of perforin is regulated by important proteins including IFN-β, IL-2, IL-6, IL-12, IL-15 and IL-21 [89, 90]. In CFS IL-2, IL-6, IL-15 and IL-21 are known to be characterised by alterations in cytokine levels, this may be a contributory factor to the decrease in cytotoxicity [91, 92].

Granzyme decrease in CFS may be attributed to decreases in perforin although correlations remain to be proven. Granzymes are serine proteases, in humans they include, granzyme A, B, H, M and K [93–96]. Granzyme A and B are the most characterised and they induce apoptosis via distraction of endoplasmic reticulum SET complex or activating of caspase 3 following cleavage of substrates as previously mentioned [97]. Granzymes are found in the extracellular fluids such as plasma, cerebrospinal fluids and synovial fluid and are therefore implicated in the regulation of inflammation [98]. The diverse role of perforin and granzymes in cell death-related pathways is paramount to immune function during infections. Hence, in CFS recurring infections may occur as a consequence of aberrations in cytotoxic activity. Importantly, NK cells employ a number of other cytotoxic pathways that may require further investigations in CFS to ascertain the exact pathway(s) that have an involvement with reduced NK cytotoxic activity.

Other factors that may affect efficient cytotoxic activity in NK cells of CFS patients may be related to cytokine production and secretion by NK cells. Cytotoxic activity may occur via IFN-γ and TNF-α. Therefore, changes in these cytokines in the CFS patients may also have a contributory role to the observed decreases in cytotoxic activity in these patients. Incidentally, cytokine studies in CFS although not NK cell-specific may have a role in reduced lysis [99, 100]. Importantly, in some CFS patients, significant increases in IFN-γ and TNF-α may indicate induction of other cell death pathways [31]. Nonetheless, this does not reflect an increase in cytotoxic activity as reduced cytotoxic activity still persists even when these pro-inflammatory cytokines are increased [31, 32]. IFN-γ is the most abundant cytokine secreted by NK cells, in particular $CD56^{bright}$ NK cells. Longitudinal assessments of cytokines in CFS patients explicitly illustrate nonconformities in the presentation of cytokine production over the cause of CFS [31]. Shifts towards pro-inflammatory cytokines such as IFN-γ and TNF-α may occur initially but dissipate in time. In most cases as we have observed in our CFS patients, an increase in IFN-γ and TNF-α occurred in coincidence with an increase in anti-inflammatory cytokine IL-10.

NK receptors in CFS

Natural killer receptors are the least investigated NK-related parameters in CFS. Currently, only one study has examined the relationship between NK receptor expression in CFS patients [65]. NK cells expression a varying number of activating and inhibitory receptors that may have stochastic presentations. It is possible to posit that failures in the regulation of the expression of these receptors can affect NK function during viral invasion. For example, elevated levels of inhibitory KIRs such as KIR3DL1 may result in decreased NK cell lyis in patients with lung cancer [101]. KIR receptors are exceedingly polymorphic and KIR3DL1 is no exception as it expresses eight different KIR3DL1 allotypes with differing sensitivities to antigen binding [102–105]. HIV and spondyloarthritis patients demonstrate high levels of KIR3DL1 [13, 106–108].

Similarly, the incidence of KIR3DS1 in some CFS patients exceeds that of non-fatigued controls [65]. A similar gene encodes KIR3DL1 and KIR3DS1 [109], suggesting a potential link between these receptors in CFS and cytotoxic activity. Certain ligands of these receptors may also be elevated in CFS patients [65]. Diversity in the KIR receptor polymorphism may generate receptors with differing haplotypes that are specific to CFS. Further studies are required to provide extensive details into the polymorphisms of KIRs in CFS patients, which may be further complicated by the heterogeneity and ambiguity of the disease presentation and symptoms.

NK gene expression studies in CFS

Natural killer gene expression or molecular studies in CFS are lacking, currently, only two studies have investigated mRNA and microRNA (miRNA) studies in CFS. Perhaps the lack of such studies relates to the cost associated and volume of blood required for the preferential isolation of NK cells. In PBMCs, expression of GZMA and GZMB is reduced in CFS patients in comparison to non-fatigued controls. GZMA and GZMB are genes for the protein granzyme A and B, respectively. These reductions may correlate with the protein production. In our previous studies, preferential examination of lytic protein genes in CFS patients revealed a significant expression in the perforin gene *PRF1* while *GZMA* and *GZMK* were significantly reduced in the CFS patients [32]. The exact cause of increase in the expression of perforin is not known, however, as perforin proteins were not measured in these CFS patients, it is difficult to predetermine an association between these *PRF1* expression and perforin protein.

MicroRNAs are non-coding small RNA molecules with regulatory roles in the expression of genes including translation repression or mRNA degradation [110, 111]. In CFS, NK cell expression of miR-10a, miR-21, miR-103, miR-106, miR-146a, miR-150, miR-17-5p, miR-191 and miR-223 are down-regulated in comparison to non-fatigued controls [112]. Most of these miRNAs have been linked to a number of cancers. An association or the role of these miRNAs in NK cell-related activities is yet to be determined nonetheless these miRNAs are attributed to a number of diseases and physiological processes. Most of these miRNAs are associated with the presentation of a number of different cancers and are involved in apoptosis, cell proliferation and development. Importantly, miR-21, miR-150 are implicated in the development of lymphocytes and thus they may have similar effects in NK cells [113, 114]. Decreases in the expression of miR-10a occur in chronic myeloid leukaemia [115]. MiR-10a preserves vascular integrity by targeting HOXA1, MAP3K7 and bTRC [116]. MiR-146a upon induction has been shown to target TNF receptor-associated factor 6 (TRAF6) and the IL-1 receptor associate kinase 1 (IRAK1) genes, and these are important in the regulation of TLRs and inflammation [117]. In many cancers the presence of miR-146a resulted in cell proliferation [118]. Bacterial antigens and pro-inflammatory cytokines stimulate the expression of miR-146a, which in turn may suppress the secretion of inflammatory cytokines [119]. Similarly, miR-21 promotes tumour growth owing to its oncogenic properties and its role in inflammation and T cell-related activities [120].

These studies on miRNAs have elucidated an important role of miRNAs in NK cells, as they regulate the expression of immune-related genes. However, these studies are

limited as they have not identified the exact miRNA target genes in CFS patients. Such studies may be instrumental in unexplained disorders such as CFS, further research, may be required to establish these links.

Implications for severe CFS patients

The results from studies on NK cells in CFS patients suggest a potential mechanism of CFS can be identified through a thorough study of NK cell-related activities. From our observations, reductions in lytic proteins, genes and further decreases in miRNA genes [31, 34, 112], cumulatively affect efficient cytotoxic activity in CFS patients. Similarly, the polymorphic alleles of the KIR receptors may not allow efficient pathogenic and antigenic targeting of the NK cell, as an overabundance in the inhibitory KIRs may abort or impede cytotoxic activity [121]. The extent immune dysfunction in subtypes of CFS patients may differ among subgroups of patients. CFS patients may have variations in the severity of their symptoms, for example a distinct subgroup of patients maybe housebound as they suffer from high levels of fatigue and CFS-related symptoms compared to other sedentary CFS patients [122]. Their severe persistent and incapacitating symptoms probably exclude these patients from CFS-related studies. Hence, we examined for the first time NK cell-related parameters including cytotoxic activity, phenotypes and KIR receptor expression in patients with severe CFS (SCFS) in comparison to sedentary or moderate CFS (MCFS) patients and non-fatigued controls. Currently, these studies have not been performed in this group of CFS patients.

NK cytotoxic activity in severe CFS patients

Cytotoxic activity of the NK cells was measured by the ability of the cells to lyse the K562 effector cells. The NK cytotoxic activity against K562 cells was significantly decreased in the MCFS and the SCFS group ($P < 0.05$) compared to the non-fatigued control group (Fig. 1). Multiple comparison tests revealed significant decreases between the SCFS patients and the control group only. There were no significant differences between the MCFS and the control group or the SCFS and the MCFS group.

NK receptors in severe CFS patients

The percentage of NK receptor expression was determined following preferential gating on isolated NK cells in a forward and side scatter plot. This was then extrapolated on to six plots for CD56 versus the six NK receptors assessed. Significant changes in NK receptors were observed in only one receptor, KIR3DL1 (CD158e) (Fig. 2). KIR3DL1 expression was significantly different between the MCFS group and the non-fatigued controls. There was a general trend of reduced receptor expression in the non-fatigued controls in comparison to the other two groups. However, most of these observations were not statistically significant (data not shown).

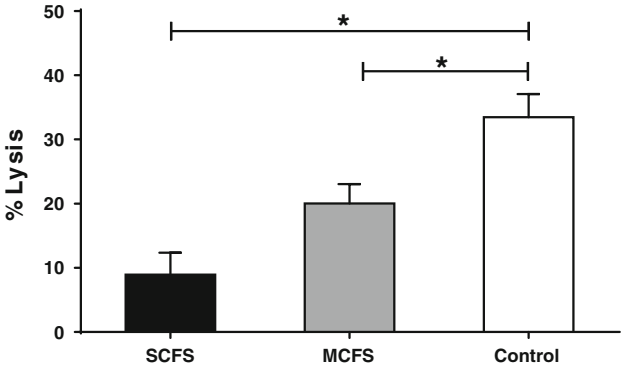

Fig. 1 Decreased NK lysis in MCFS and SCFS group compared to a non-fatigued control group. The percent lysis of NK cells in each group is represented above where the *white bar* represents the results from the non-fatigued control group and the *black bar* represents the SCFS group. *Asterisk* denotes statistical significance where $P < 0.05$ and data is represented as the mean ± SEM

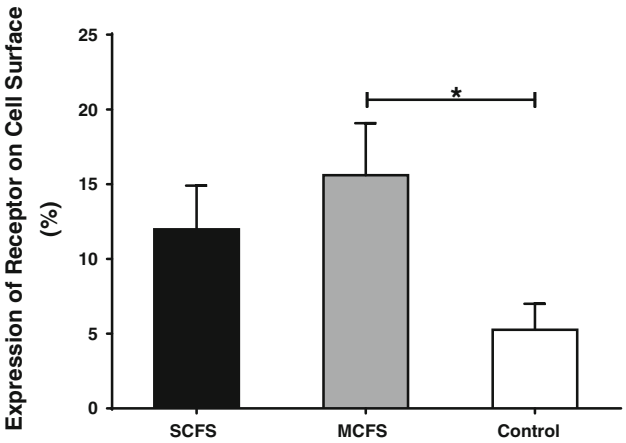

Fig. 2 Expression of KIR3DL1 in SCFS, MCFS and a non-fatigued control group. The above bar graph is based on the flow cytometric analysis of KIR3DL1. This was the only receptor that was significantly ($P < 0.05$) increased in the MCFS group in comparison to the SCFS and non-fatigued controls. There was no significant difference between the SCFS group and the MCFS group. *Asterisk* denotes statistical significance where $P < 0.05$ and data is represented as the mean ± SEM

NK cytokines in severe CFS patients

In the present study, plasma cytokines were investigated in SCFS, MCFS and non-fatigued controls, where a significant increase in the plasma pro-inflammatory cytokines IFN-γ and TNF-α were observed in the SCFS patients. Additionally, IL-4 was significantly increased in the SCFS group (Fig. 3).

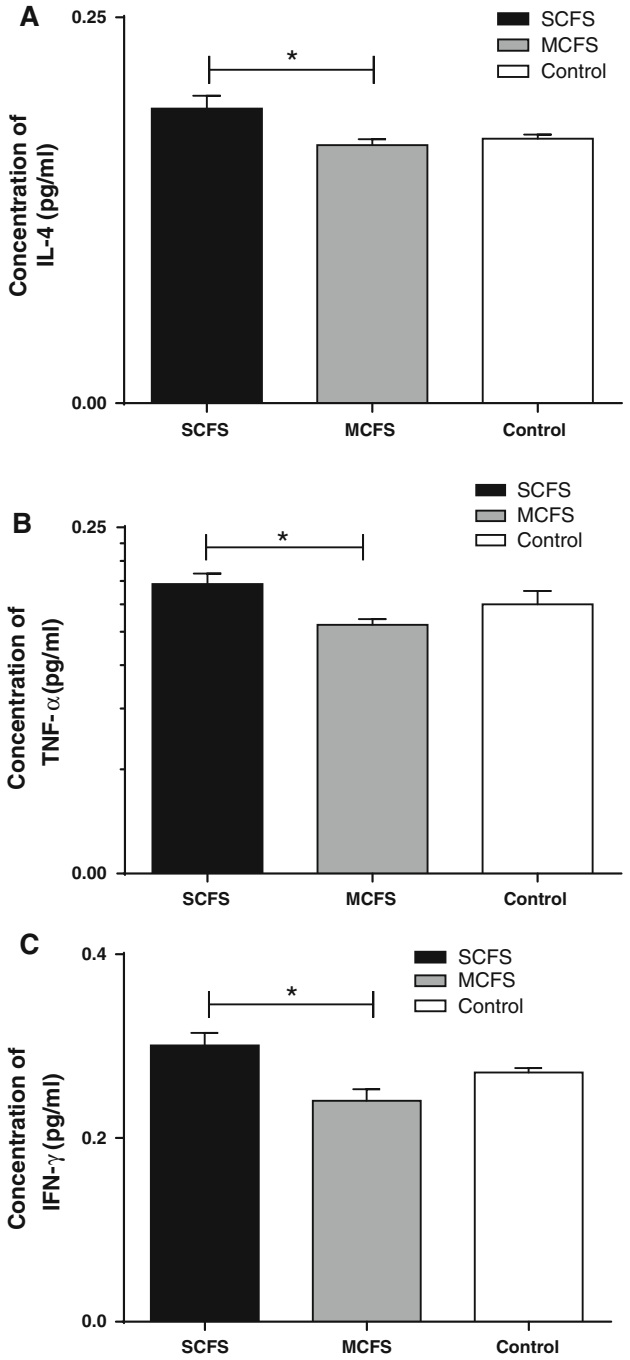

Fig. 3 Plasma Cytokines in SCFS, MCFS and a non-fatigued control group. The above bar graph is based on enzyme-linked immunosorbent assay (ELISA) assessments of seven plasma cytokines (IFN-γ, IL-1b/IL-1F2, IL-2, IL-4, IL-17, IL-6 and TNF-α). IL-4 (**a**), TNF-α (**b**) and IFN-γ (**c**) were significantly increased ($P < 0.05$) in the SCFS group in comparison to the MCFS and non-fatigued controls. *Asterisk* denotes statistical significance where $P < 0.05$. Results are represented as the mean ± SEM

Conclusion

This preliminary study is the first to examine and report immunological disparities in severely affected CFS patients characterised by significant decreases in NK lysis and increases in KIR3DL1, IL-4, TNF-α and IFN-γ.

The decreases in cytotoxic activity observed in the SCFS and MCFS group were consistent with previous CFS NK studies [34, 37, 69]. These NK disparities likely occur as a consequence of paucities in lytic proteins including perforin and granzymes and differential expression of their genes in some CFS patients [37, 85]. These molecules are involved in the granule-dependent cytotoxic pathway. Perforin is a necessary component of this pathway as it facilitates the entry of the granzymes into the target cell. In the target cell, granzymes activate caspases, mitochondria-related apoptosis and reactive oxygen species, which induce apoptosis [123]. Importantly, mice deficient in perforin experience a substantial loss in cytotoxic activity. Reduced cytotoxic activity permits the recurrence and prolonged survival of various infections in the body possibly explaining the persistence of flu-like symptoms in the CFS patients. As correlations exist between cytotoxic activity and perforin in CFS patients, a similar incidence may present itself in SCFS patients perhaps at a more severe rate in comparison to the moderately affected CFS population. Nonetheless, further confirmatory studies are now required.

Significant increases in the expression of inhibitory KIRs may correspond to the reduced NK cell lysis [124]. Specifically, the significant increase in KIR3DL1 may be related to decreases in NK cell cytotoxicity of infectious cells with Class I HLA expression [101]. KIR3DL1 is a highly polymorphic inhibitory NK receptor and polymorphisms in its gene results in the generation of eight different KIR3DL1 allotypes that may be classified as having high, intermediate or no surface expression with similar affinity to bind antigens [102, 104, 105]. It associates with antigens expressing HLA-B and having Bw4 specificity [125]. Variations in the allotypes determine the response of the KIR3DL1 to pathogens at the cell surface [104]. For example expression of an inactivated KIRD3DL1 phenotype at the cell surface maybe subverted by ligands from viral pathogens and this may be related to certain disease presentations [126]. Similarly, polymorphisms within the HLA-Bw4 may undermine recognition by KIR3DL1 [127]. Increases in KIR3DL1 have been associated with diseases such as HIV and spondyloarthritis [66, 128]. KIR genes have previously been investigated in CFS patients where frequency of KIR3DS1 was significantly elevated in the CFS patients in comparison to the non-CFS group [65]. Similarly, the incidence of KIR3DL1 and KIR3DS1 without HLA-ligand and HLA Ile80, respectively, was higher among the CFS patients [65]. KIR3DL1 and KIR3DS1 are encoded by the same gene [109], hence, these observations implicate possible compromises to the genetic framework that confers atypical properties on these receptors and their allotypes inadvertently compromising cytotoxic activity.

The increase in IFN-γ did not correlate with an increase in cytotoxic activity as cytotoxicity was reduced in the CFS patients thus indicating that other cell types or cytokines such as TNF-α may have contributed to the overall increase in plasma IFN-γ levels. The results on the cytokine studies further highlight profound compromises in the immune function of SCFS patients in comparison to the MCFS patients. IFN-γ and TNF-α activate macrophages and $CD8^+$T cells and provoke T helper 1-related immune responses [129]. Persistent T cell and macrophage activation, decreased NK activity and impaired perforin function is a hallmark of hemophagocytic lymphohistiocytosis [130]. Hence, atypical immune activation may exist among SCFS patients. Perhaps, cell-specific cytokine assessments may provide superior in-depth analysis of cytokines in CFS patients. Although, we have attempted to provide and highlight cytokines in plasma this may still not be representative of the cytokine profile in CFS patients as the source of most of these cytokines were not examined in this study and thus remains unknown. Nonetheless, this is the first study to report on cytokines in SCFS patients and may serve as a platform for further studies.

Contrary to previous studies, the present study did not demonstrate any significant reductions or changes in NK phenotypes. CFS is a heterogeneous disease and different subgroups of CFS patients may potentially express different distributions in immune cell phenotypes [55]. Incidentally, we have previously shown that alterations in NK phenotypes are not consistent overtime but fluctuate and are therefore poor indicators of immune function in CFS patients [31]. Reduced NK lysis with concomitant increases in KIR3DL1 and cytotoxic-related cytokines is suggestive of impairments in the NK cell cytotoxic pathways, in particular, the granule-dependent and -independent pathways. Further studies are now required to elucidate the mechanisms of these pathways in the CFS patients with varying degrees of symptom severity. Importantly, KIR receptors may be important biomarkers for the diagnosis of CFS following thorough validatory studies to determine their use in CFS diagnosis.

Conflict of interest E.W. Brenu, S.L. Hardcastle, G.M. Atkinson, Mieke L. van Driel, Sanne Kreijkamp-Kaspers, K.J. Ashton, D.R. Staines, S.M. Marshall-Gradisnik declare that they have no conflict of interest.

Informed consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2005. Informed consent was obtained from all patients for being included in the study.

Animal studies No animal studies were carried out by the authors for this article.

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