

Impaired calcium mobilization in natural killer cells from chronic fatigue syndrome/myalgic encephalomyelitis patients is associated with transient receptor potential melastatin 3 ion channels

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Summary

Transient receptor potential melastatin subfamily 3 (TRPM3) ion channels play a role in calcium (Ca^{2+}) cell signalling. Reduced TRPM3 protein expression has been identified in chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) patients. However, the significance of TRPM3 and association with intracellular Ca^{2+} mobilization has yet to be determined. Fifteen CFS/ME patients (mean age 48.82 ± 9.83 years) and 25 healthy controls (mean age 39.2 ± 12.12 years) were examined. Isolated natural killer (NK) cells were labelled with fluorescent antibodies to determine TRPM3, CD107a and CD69 receptors on $\text{CD56}^{\text{dim}}\text{CD16}^{\text{+}}$ NK cells and $\text{CD56}^{\text{bright}}\text{CD16}^{\text{dim/-}}$ NK cells. Ca^{2+} flux and NK cytotoxicity activity was measured under various stimulants, including pregnenolone sulphate (PregS), thapsigargin (TG), 2-aminoethoxydiphenyl borate (2APB) and ionomycin. Unstimulated $\text{CD56}^{\text{bright}}\text{CD16}^{\text{dim/-}}$ NK cells showed significantly reduced TRPM3 receptors in CFS/ME compared with healthy controls (HC). Ca^{2+} flux showed no significant difference between groups. Moreover, PregS-stimulated $\text{CD56}^{\text{bright}}\text{CD16}^{\text{dim/-}}$ NK cells showed a significant increase in Ca^{2+} flux in CFS/ME patients compared with HC. By comparison, unstimulated $\text{CD56}^{\text{dim}}\text{CD16}^{\text{+}}$ NK cells showed no significant difference in both Ca^{2+} flux and TRPM3 expression. PregS-stimulated $\text{CD56}^{\text{dim}}\text{CD16}^{\text{+}}$ NK cells increased TRPM3 expression significantly in CFS/ME, but this was not associated with a significant increase in Ca^{2+} flux. Furthermore, TG-stimulated $\text{CD56}^{\text{dim}}\text{CD16}^{\text{+}}$ NK cells increased K562 cell lysis prior to PregS stimulation in CFS/ME patients compared with HC. Differential expression of TRPM3 and Ca^{2+} flux between NK cell subtypes may provide evidence for their role in the pathomechanism involving NK cell cytotoxicity activity in CFS/ME.

Keywords: cell surface molecules, inhibitory/activating receptors, natural killer cells

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Introduction

Chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) is a complex illness presenting with debilitating post-exertional malaise accompanied by other neurological, immunological, autonomic and ion transport impairments [1,2]. The most consistent biological evidence reported in CFS/ME has been reduced natural killer (NK) cell cytotoxicity (reviewed by Stayer *et al.* [3]).

NK cells belong to the innate immune system and are distinguished into two main subsets known as $\text{CD56}^{\text{bright}}\text{CD16}^{\text{dim/-}}$ NK cells and $\text{CD56}^{\text{dim}}\text{CD16}^{\text{+}}$ NK

cells. $\text{CD56}^{\text{bright}}\text{CD16}^{\text{dim/-}}$ NK cells are predominately immunosurveillance cells with potent cytokine production, whereas $\text{CD56}^{\text{dim}}\text{CD16}^{\text{+}}$ NK cells are cytotoxic and kill infected, tumour or 'missing self' cells [4].

Activated NK cells increase CD69 receptors and may be modulated by protein kinase C (PKC) and protein tyrosine kinases [5]. NK cells from CFS/ME patients have been reported to have a significant reduction in CD69 receptors after stimulation [6]. When NK cells come into contact with virus-infected or tumour cells, an immune synapse is

formed and degranulation is activated. Degranulation is detected when NK cells increase the lysosome-associated membrane protein-1 (LAMP-1, CD107a) receptor [7]. NK cells require Ca^{2+} to regulate cellular functions, which play a major role in signalling pathways that are essential for development of immune synapse formation, cytokine production and cytotoxic activity [8].

Transient receptor potential (TRP) ion channels modulate ion entry in the cell plasma membrane, and are expressed differentially on tissues throughout the body. Their dysfunction has been associated with several diseases, such as cancer, glomerulosclerosis and polycystic kidney disease [9]. Transient receptor potential melastatin 3 ion channels (TRPM3) are transmembrane ion channels belonging to the melastatin subfamily of TRP cation ion channels [10,11]. There are seven isoforms identified in the human TRPM3 gene; in particular, the TRPM3 α 2 isoform has been characterized as having a Ca^{2+} and Mg^{2+} ion-conducting pore [12,13]. TRPM3 ion channels are expressed in various tissues, predominantly in sensory neurones, kidney, brain, pituitary gland and pancreas. While expressed ubiquitously in mammalian cells, the roles and functions of TRPM3 have yet to be determined in immune cells [10]. TRPM3 ion channels are activated selectively by a potent neurosteroid, pregnenolone sulphate (PregS) [14]. Pregnenolone is synthesized from cholesterol by many tissues, including adrenal glands, brain and skin. These tissues have been reported to express TRPM3 [10]. Physiologically, PregS plays a role in both the central nervous system and immune system, which include improvement of memory and cognitive function, enhanced neuronal myelination, activation of neurotransmitter-gated channels, modulation of glutamate–nitric oxide–guanosine 3',5'-(cyclic) phosphate pathways, regulation of insulin secretion and the management of noxious pain [14,15].

Recently, TRPM3 ion channels were identified on NK cells and B cells from CFS/ME patients [16], and these cells were shown to have significantly reduced expression of the TRPM3 receptor on the cell surface compared with healthy controls (HC) [17]. However, the role of TRPM3 in terms of receptor number and Ca^{2+} mobilization following stimulants, such as PregS on NK cell function, has not been investigated in CFS/ME patients. Therefore, the objective of this study was to determine the effects of PregS on TRPM3, including CD69 and CD107a receptor expression in NK cells. Additionally, we investigated intracellular Ca^{2+} mobilization and cytotoxic activity of NK cells after PregS treatment in HC and CFS/ME patients.

Materials and methods

Participants

CFS/ME patients meeting the Fukuda definition were included into the study, along with HC [2]. All participants

were recruited between the ages of 18 and 65 years, with no previous history of smoking or chronic disease (auto-immune diseases, cardiac diseases and primary psychological disorders). Participants were further excluded if pregnant, breast feeding or taking hormone therapy and pharmaceutical immunoregulatory medications. Alternatively, participants on immunoregulatory medications were included into the study if medications were discontinued 2 weeks prior to their blood collection.

Participants living in the South East Queensland region of Australia donated 80 ml of blood between 8:00 a.m. and 10:30 a.m. Seventy ml of blood was used to isolate NK cells for further study, while the remaining 10 ml was used for routine pathology screening to help determine any underlying illness. Pathology results included a full blood cell count, electrolytes, erythrocyte sedimentation rate, C-reactive protein, dehydroepiandrosterone, adrenocorticotropic hormone and cortisol. All participants completed a self-reported questionnaire on their current symptoms, which also included the Short-Form Health Survey (SF-36) and WHO Disability Assessment Schedule 2.9 (WHO DAS 20.0) to assess participants' physical health.

Drugs and antibodies

In-vitro assays consisted of using the following drugs at a final concentration of 1 μM ionomycin, 50 μM 2-aminoethoxydiphenyl borate (2-APB), 1.42 μM thapsigargin (TG) and 1.35 μM PregS. All drugs were purchased from Bio-Techne (Tocris Bioscience, Sussex, UK) except ionomycin, which was purchased from Sigma (Mansfield, Victoria, Australia). Antibodies CD3, CD56, CD16, CD69 and CD107a were purchased from Beckon Dickinson Bioscience (Miami, FL, USA). TRPM3 primary and secondary antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Isoforms were used to determine negative cell populations. TRPM3 primary antibody was blocked by peptide amino acids that bind to the 816-897aa of TRPM3 of human origin (Accession number Q9HCF6) and binds to the extracellular domain to determine non-specific binding.

NK cells

Peripheral blood mononuclear cells (PBMCs) were isolated from ethylenediamine tetraacetic acid (EDTA) whole blood by centrifugation over a density gradient medium (Ficoll; GE Healthcare, Pittsburgh, PA, USA), followed by magnetic isolation for unlabelled NK cells using EasySep, as described by the manufacturer's instructions. Isolated NK cells from PBMCs were determined to be $90.4\% \pm 3.82$ purity for CFS/ME patients and $91.6\% \pm 5.61$ for HC. Isolated NK cells were identified as $\text{CD56}^{\text{bright}}\text{CD16}^{-/\text{dim}}$ and $\text{CD56}^{\text{dim}}\text{CD16}^{+}$ NK cells.

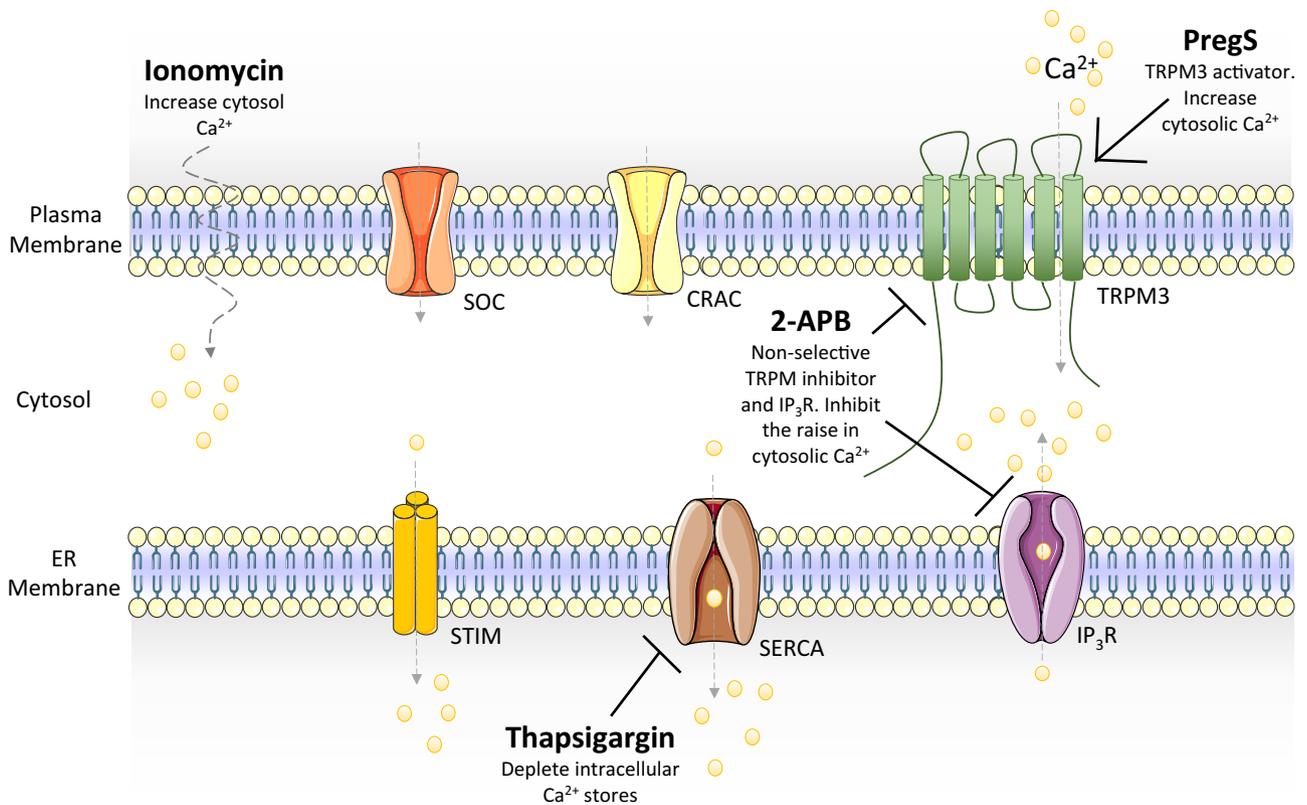


Fig. 1. Systematic representation of calcium ions mobilization. Ionomycin is an ionopore that allows extracellular calcium ion into the cytosol. 2-APB is a non-selective TRPM and IP₃R receptor, whereas thapsigargin inhibits intracellular calcium stores replenishment. ER = endoplasmic reticulum; SOC = store-operated calcium channel; CRAC = calcium release activated channel; TRPM3 = transient receptor potential melastatin subfamily 3; STIM = stromal interaction molecule; SERCA = sarco/endoplasmic reticulum Ca²⁺-ATPase; IP₃R = inositol trisphosphate receptor; PregS = pregnenolone sulphate; 2-APB = 2-aminoethoxydiphenyl borate; Ca²⁺ = calcium.

TRPM3, CD69 and CD107a surface expression on NK cells

TRPM3 expression on resting NK cell subsets was identified as described previously [16]. Briefly, NK cells were labelled with CD3, CD56, CD16 and primary TRPM3 antibodies for 30 min at room temperature. NK cells were washed and stained with TRPM3 secondary antibody for 30 min. Stimulated NK cells were assessed further in the presence of PregS, ionomycin, 2APB + PregS and TG + PregS for 4 h at 37°C. Cells were stained with CD69, CD107a and TRPM3 primary antibody for 30 min to determine CD69, CD107a and TRPM3 receptor expression on CD56^{bright}CD16^{dim/-} NK cells and CD56^{dim}CD16⁺ NK cell subpopulations. True cell counting beads were used to calculate NK cell concentration as well as absolute cell counts and was determined using the manufacturer's instructions outlined in the following formula:

$$\begin{aligned} & \frac{\text{number of events in cell region}}{\text{number of events in bead region}} \\ & \times \frac{\text{number of beads/test}}{\text{test volume}} \\ & \times \text{dilution factor} \\ & = \text{concentration of cell population} \end{aligned}$$

Cytoplasmic Ca²⁺ in NK cells

Ca²⁺ flux was performed as described previously [16]. Briefly, Ca²⁺ mobilization in NK cells was measured in the presence of ionomycin, 2-APB and TG. Ionomycin is an ionopore that raises cytoplasmic Ca²⁺ levels independently of activated receptors, whereas 2-APB and TG inhibit Ca²⁺-dependent receptors on the endoplasmic reticulum (ER). 2-APB prevent the release of intracellular Ca²⁺ stores compared to TG, which inhibit the replenishment of the Ca²⁺ into intracellular stores (Fig. 1). Furthermore, assessment of Ca²⁺ influx-dependent TRPM3 receptors was determined using PregS. Isolated NK phenotypes were stimulated with PregS. Following this, NK cells were preincubated with either TG or 2APB prior to PregS stimulation. Cells were stimulated for 5 min and recorded in real time on flow cytometry.

NK cytotoxic activity

NK cell cytotoxicity baseline measurements were performed using 25 : 1, 12 : 1, 6 : 1 and 1 : 1 effector to target ratios, as described previously [18]. NK cell cytotoxicity activity was measured indirectly through the percentage of

apoptotic K562 cells. The NK cells were stimulated for 20 min in the presence of ionomycin, TG, 2APB and PregS or a combination of TG + PregS and 2APB + PregS. Cells were washed and plated with K562 cells at a 1 : 1 ratio, followed by incubation for 4 h at 37°C. K562 cells were stained with annexin V and 7-amino-actinomycin D (7-AAD) to determine K562 cell viability. K562 cell viability

was determined into four stages; live/viable K562 cells (annexin V⁻ 7AAD⁻), K562 cells undergoing early stage apoptosis (annexin V⁺ 7AAD⁻), late stage apoptosis (annexin V⁺ 7AAD⁺) and dead K562 cells (annexin V⁻ 7AAD⁺). K562 cell death was calculated as described previously [18] and outlined below.

$$\text{Events of K562 cells in the presence of NK cells} \left[\frac{\text{early stage apoptosis} + \text{late stage apoptosis} + \text{dead K562}}{\text{All K562 cells}} \right]$$

$$\text{Subtract events of K562 cells only} \left[\frac{\text{early apoptosis} + \text{late apoptosis} + \text{dead K562}}{\text{All K562 cells}} \right] \text{ multiple by } 100 = \% \text{ K562 cell death}$$

Data and statistical analyses

Data were exported from FacDiva version 8.1 and analysed using FlowJo software version 10 (TreeStar, Inc., Ashland, OR, USA). Data were quantified using SPSS version 22.2 software (IBM Corp., Armonk, NY, USA). Multivariate analysis of variance (MANOVA) was performed to determine TRPM3 receptor expression in various stimulation conditions between HC and CFS/ME groups, as well as within each group. The Mann-Whitney statistical *U*-test was used to determine the specific relationship effects between each stimulant (PregS, TG + PregS and 2APB + PregS) on TRPM3, CD69 and CD107a surface expression on NK cell subsets from CFS/ME patients and HC. The Gaussian smoothing method was applied on the Ca²⁺ flux response curve, prior to analyses of area under the curve (AUC), time of peak and slope. NK cell cytotoxic activity was measured by the percentage of K562 cell deaths as described by Aubry *et al.* [18]. Statistical significance was reported at *P* < 0.05.

Results

Participants

A total of 40 participants were included in this investigation, 25 of which were HC and 15 were CFS/ME. The participants' demographic and standard serological results were recorded (Table 1). There were no significant differences in gender, body mass index (BMI), full blood counts, electrolytes, stress hormones or inflammatory markers between HC and CFS/ME. There was a significant between-group difference for age, as CFS/ME patients were significantly older than HC.

CFS/ME patients reported greater impairment compared with HC in all SF-36 subscales (lower scores indicate greater impairment; *P* < 0.05) and WHO DAS 2.0 subscales (higher scores indicate greater impairment; *P* < 0.05), as shown in Table 1. Significantly reduced SF-36 scores and elevated WHO DAS 2.0 scores have been observed previously in other CFS/ME studies [19–21].

TRPM3 expressed on CD56^{bright}CD16^{-dim} NK cells

CFS/ME had significantly reduced expression for TRPM3 receptors without stimulation in CD56^{bright}CD16^{-dim} NK cells compared with HC (Fig. 2a–c). Similarly, TG-stimulated CD56^{bright}CD16^{-dim} NK cells had depleted intracellular Ca²⁺ stores and significantly reduced TRPM3 receptor expression in CFS/ME compared with HC (Fig. 2b). Overall, CD56^{bright}CD16^{-dim} NK cells from both groups (CFS/ME and HC) had increased TRPM3 receptors after depletion of intracellular Ca²⁺ stores with TG and/or in combination with activating TRPM3 receptor with PregS, when compared without stimulation (Fig. 2b). Furthermore, inhibiting inositol trisphosphate receptor (IP₃R) with 2APB and/or in combination with activating the TRPM3 receptor with PregS in CD56^{bright}CD16^{-dim}TRPM3⁺ NK cells was not significantly different between or within each HC and CFS group (Fig. 2c).

TRPM3 expressed on CD56^{dim}CD16⁺ NK cells

There was no significant between-group difference in TRPM3 expression without stimulation of CD56^{dim}CD16⁺ NK cells. However, there was a significant between-group difference (CFS/ME and HC) in TRPM3 expression following PregS stimulation (Fig. 2d). Interestingly, within the HC group there was a significant increase in TRPM3 ion channels on PregS-stimulated CD56^{dim}CD16⁺ NK cells compared without stimulation. The CFS/ME group showed no significant difference in TRPM3 ion channel numbers in CD56^{dim}CD16⁺ NK cells after PregS stimulation. In addition, ionomycin-stimulated CD56^{dim}CD16⁺ NK cells within HC and CFS/ME groups demonstrated a significant increase in TRPM3 ion channels (Fig. 2d). Similarly, CD56^{dim}CD16⁺TRPM3⁺ NK cells within both groups increased TRPM3 receptors with TG + PregS, TG and ionomycin when compared without stimulation. By comparison, no significant difference was observed after 2APB or 2APB + PregS stimulation between or within each of the groups (HC and CFS/ME) (Fig. 2f).

Table 1. Participants' demographic and serological results

Category	Item	HC	CFS	<i>P</i> -value
General demographics	<i>n</i>	25	15	
	Gender			
	Female (% , <i>n</i>)	68% (17)	70.6% (12)	
	Male (% , <i>n</i>)	32% (8)	29.4% (5)	
	Age (years)	39.20 ± 12.124	48.824 ± 9.838	0.01
	BMI (kg/m ²)	25.387 ± 4.383	25.602 ± 4.945	0.894
Illness demographics	SF-36 score			
	Physical functioning (%)	100 ± 0.000	59.166 ± 27.289	0.016
	Role limitations due to physical health (%)	97.5 ± 7.905	2.083 ± 7.216	0.000
	Role limitations due to emotional problems (%)	96.66 ± 10.540	50 ± 46.056	0.000
	Energy/fatigue (%)	73 ± 12.292	20.833 ± 16.899	0.000
	Emotional well-being (%)	83.2 ± 7.495	63 ± 10.531	0.000
	Social functioning (%)	94.75 ± 11.453	28.125 ± 25.497	0.000
	Pain (%)	97.5 ± 5.270	43.75 ± 29.435	0.000
	General health (%)	81.5 ± 12.258	34.166 ± 15.930	0.000
	WHO DAS 2.0 (%)	0.069 ± 0.218	41.078 ± 16.216	0.000
Serology report	Full blood count			
	WBC (×10 ⁹ /l)	5.736 ± 1.241	6.212 ± 1.162	0.218
	Lymphocytes (×10 ⁹ /l)	1.865 ± 0.604	1.967 ± 0.468	0.560
	Monocytes (×10 ⁹ /l)	0.314 ± 0.097	0.332 ± 0.090	0.547
	Neutrophils (×10 ⁹ /l)	3.363 ± 0.831	3.708 ± 0.961	0.222
	Eosinophils (×10 ⁹ /l)	0.160 ± 0.088	0.182 ± 0.128	0.517
	Basophils (×10 ⁹ /l)	0.029 ± 0.019	0.027 ± 0.014	0.609
	Platelets (×10 ⁹ /l)	263.000 ± 68.512	244.588 ± 78.167	0.424
	Haemoglobin (g/l)	138.000 ± 11.384	142.941 ± 10.556	0.163
	Haematocrit (%)	0.413 ± 0.031	0.428 ± 0.029	0.134
	RBC (×10 ¹² /l)	4.670 ± 0.368	4.769 ± 0.371	0.395
	MCV (fL)	88.560 ± 3.927	89.588 ± 3.355	0.383
	Electrolytes			
	Sodium (mmol/l)	136.600 ± 2.345	129.325 ± 33.152	0.278
	Potassium (mmol/l)	4.116 ± 0.405	4.125 ± 0.218	0.935
	Chloride (mmol/l)	102.560 ± 1.960	102.313 ± 2.089	0.703
	Bicarbonate (mmol/l)	26.280 ± 2.011	27.563 ± 2.097	0.057
	Anion gap (mmol/l)	7.800 ± 1.893	7.750 ± 2.352	0.941
	Inflammatory markers			
	ESR (mm/h)	13.240 ± 9.854	12.882 ± 8.950	0.905
	CRP (mg/l)	1.973 ± 2.165	2.417 ± 3.620	0.633
	Stress hormones			
	DHEA sulphate (µmol/l)	4.132 ± 2.129	3.733 ± 2.230	0.576
	ACTH (ng/l)	17.880 ± 8.303	15.625 ± 6.386	0.361
	Cortisol (nmol/l)	358.640 ± 132.282	286.067 ± 89.850	0.068

Data shown are mean ± standard deviation. HC = healthy controls; CFS = chronic fatigue syndrome; BMI = body mass index; WBC = white blood cell; RBC = red blood cell; MCV = mean corpuscular volume; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DHEA = dehydroepiandrosterone; ACTH = adrenocorticotropic hormone. WHO DAS = World Health Organization Disability Assessment Schedule; SF-26 = Short-Form Health Survey. Bold type indicates statistical significance ($P < 0.05$).

Activation markers on NK cell subsets

There was no significant difference in either CD56^{bright} CD16^{-dim} or CD56^{dim}CD16⁺ NK cells in CD107a and CD69 receptors between groups (data not shown). However, both NK cell subsets within groups showed no significant difference in CD69 receptor numbers between unstimulation and PregS. There was a significant reduction of CD69 receptor after TG and TG + PregS stimulations (Fig. S1). CD56^{dim}CD16⁺ NK cells within groups showed a

further reduction of CD69 receptor after 2APB and ionomycin compared without stimulation.

Ca²⁺ mobilization in NK cell subsets was determined using FlowJo, as shown in Fig. 3. Each subset Ca²⁺ response curve was reported as peak time (peak *t*) to determine the time for a reaction to reach its maximum. Slope is determined as follows: a positive slope implies that the *y*-axis value is increasing over the time-period, while a negative value implies that it is decreasing. The AUC is

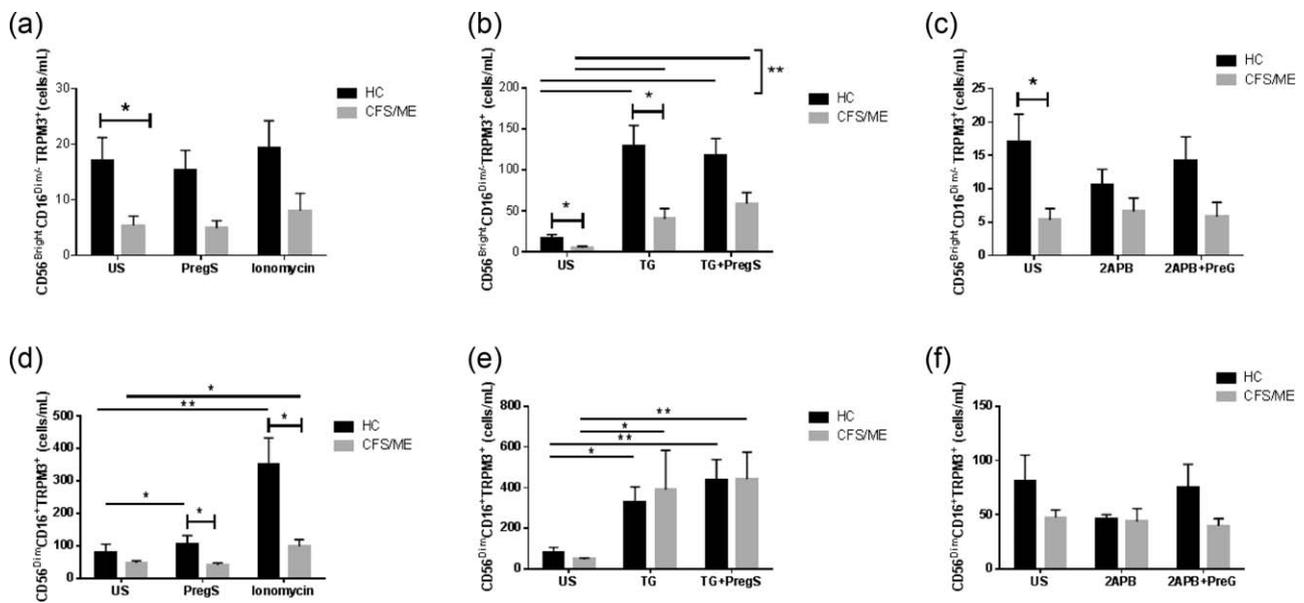


Fig. 2. Natural killer (NK) cell TRPM3 expression in HC and CFS/ME patients. CD56^{bright}CD16^{dim/-} NK cells expressed TRPM3 as cells/ml under various conditions. (a) Unstimulated PregS and ionomycin. (b) TG and TG + PregS. (c) 2APB and 2APB + PregS. CD56^{dim}CD16⁺ NK cells expressed TRPM3 as cells/ml under various conditions. (d) PregS and ionomycin. (e) TG and TG + PregS. (f) 2APB and 2APB + PregS. Data are represented as mean \pm standard error of the mean. Asterisks (*) and (**) represent statistical significance at $P < 0.05$ and $P < 0.01$, respectively. US = unstimulated; PregS = pregnenolone sulphate; TG = thapsigargin; HC = healthy controls; 2-APB = 2-aminoethoxydiphenyl borate; CFS/ME = chronic fatigue syndrome/myalgic encephalomyelitis. [Colour figure can be viewed at wileyonlinelibrary.com]

calculated to determine the duration and magnitude of Ca²⁺ flux, which is represented as a single number.

Intracellular Ca²⁺ mobilization

CD56^{bright} CD16^{dim/-} NK cell Ca²⁺ flux showed significantly increased AUC in CFS/ME compared with controls after PregS (Fig. 4a). There was no significant difference in

the AUC in CD56^{dim}CD16⁺TRPM3⁺ NK cells (Fig. 4b). Overall, within both groups there was an increase in AUC after PregS stimulation compared with no stimulation.

NK cytotoxic activity

NK cells demonstrated significantly increased cytotoxic activity when stimulated with TG + PregS in CFS/ME

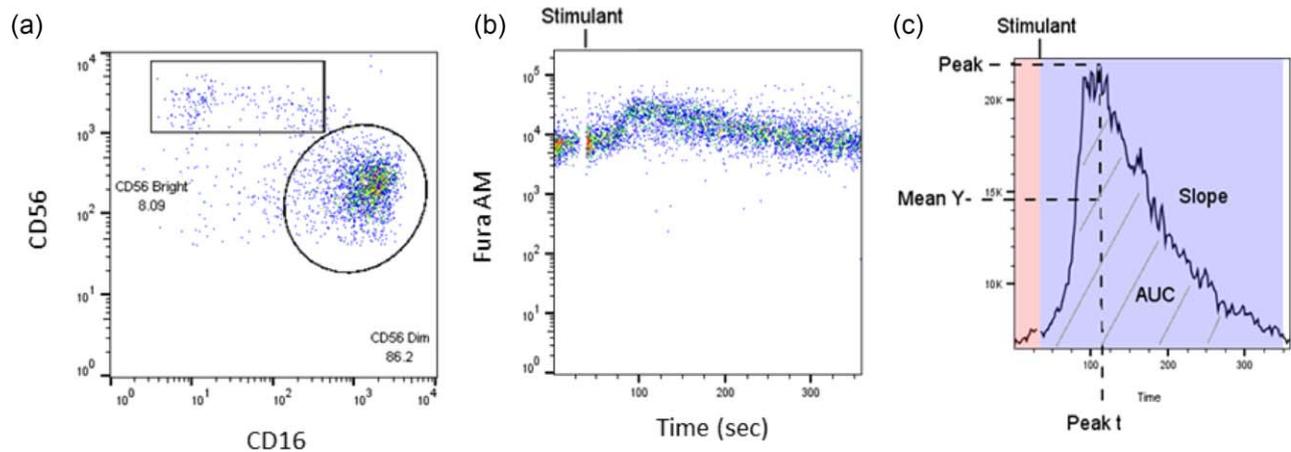


Fig. 3. FlowJo analysis of Ca²⁺ mobilization response in isolated natural killer (NK) cells. (a) Gating strategies for CD56^{bright}CD16^{dim/-} NK cells and CD56^{dim}CD16⁺ NK cells are shown as a pseudocolour dot-plot. (b) NK cell Ca²⁺ flux is response over time, and displayed as a pseudocolour dot-plot. (c) Summary of the kinetic analysis of the Ca²⁺ flux in NK cells. Peak t is the time that the maximum y -axis value occurred for the specific time range noted. Peak is the magnitude of the y -axis value at its maximum for the specific time range noted. The mean of the y -axis (mean Y) value is for the time range noted. The slope is the gain or loss of intensity over the duration of the time range for the calculated linear regression line of the data in this range. The area under the curve (AUC) is indicated by the grey stripes. Background of the calcium curve is shaded in pink. Post-stimulant calcium response curve is shaded in purple.

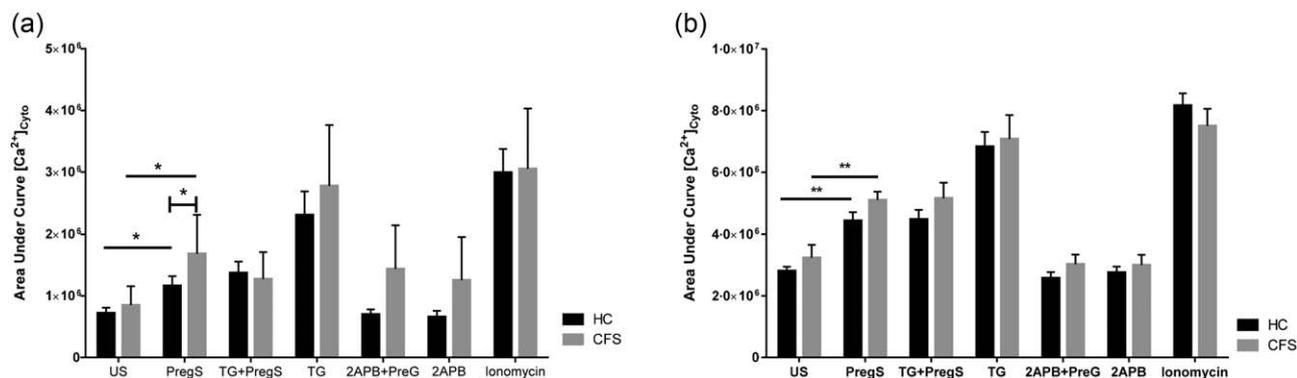


Fig. 4. Cytoplasmic calcium in natural killer (NK) cells from HC and CFS/ME patients. (a) CD56^{bright} CD16^{dim/-} NK cell calcium flux response area under the curve. (b) CD56^{dim} CD16⁺ TRPM3⁺ NK cell calcium flux response area under the curve. Data are represented as mean \pm standard error of the mean. Asterisks (*) and (**) represent statistical significance at $P < 0.05$ and $P < 0.01$, respectively. Abbreviations: US = unstimulated; PregS = pregnenolone sulphate; TG = thapsigargin; HC = healthy controls; CFS/ME = chronic fatigue syndrome/myalgic encephalomyelitis.

compared with the HC group. No significant between-group differences were seen with PregS, ionomycin and 2APB (Fig. 5).

Discussion

Previous investigations have reported significant reductions in NK cell cytotoxic activity in CFS/ME patients, and the current investigation supports those findings [16]. The current investigation also confirms our previous results of significantly reduced TRPM3 receptors on NK cells as well as significantly reduced intracellular Ca²⁺ mobilization in isolated NK cells [16]. The current investigation showed inhibition of the ER Ca²⁺/ATPase pump and depletion of intracellular Ca²⁺ stores followed by PregS-activated TRPM3 increased cytotoxic activity in NK cells from CFS/ME patients (Fig. 6).

TRPM3 receptors are constitutively open [10,12,22] and are activated by steroids [15], hypotonic cell swelling and Ca²⁺ store depletion [10], but these findings are inconsistent [22]. Our results support increased TRPM3 surface protein expression when intracellular Ca²⁺ stores are depleted through inhibition of the ER Ca²⁺/ATPase pump when activated by PregS.

Under no stimulation, CD56^{bright} CD16^{dim/-} TRPM3⁺ NK cells from CFS/ME demonstrated significantly lower TRPM3 surface expression compared with HC, which is consistent with previous findings [16]. TRPM3 can be activated by PregS concentration in the range of 0.1–5 μ M, corresponding to physiological PregS serum concentrations [23,24]. At 1.35 μ M PregS (within physiological range), CD56^{bright} CD16^{dim/-} TRPM3⁺ NK cells did not change between groups. However, these cells increased intracellular Ca²⁺ significantly compared with HC. In comparison, PregS-stimulated CD56^{dim} CD16⁺ NK cells increased TRPM3 receptor expression significantly and may not be associated with Ca²⁺ flux in CFS/ME patients. This is

based on the assumption that an increase in TRPM3 receptor should result in an increase in cytoplasmic Ca²⁺ levels. A possible rationale for this finding may be that TRPM3 ion channels expressed on CD56^{dim} CD16⁺ NK cells have impaired Ca²⁺ flux function, which is compensated by increasing their surface expression. Moreover, it may be suggested that single nucleotide polymorphisms (SNPs) identified in TRPM3 genomic DNA from PBMCs and NK cells in CFS/ME patients result in impaired protein expression and reduced TRPM3 Ca²⁺ function [17]. Importantly, when Ca²⁺ influx is prevented or reduced, an immunological synapse is not formed, preventing the formation of mitotic spindles for cytoskeleton movement to facilitate the secretory vesicles leading to abnormal lysis of target cells [25]. The deficits identified above may thus contribute to impaired lysis of CFS/ME NK cells.

Interestingly, our study supports the finding that ionomycin alone does not improve NK cytotoxic activity, but it elevates TRPM3 expression on CD56^{dim} CD16⁺ NK cells compared with HC. TRPM3 expression is increased when ionomycin raises cytosolic Ca²⁺ through phospholipase C-dependent mobilization of Ca²⁺ stores [26]. Prolonged treatment with ionomycin has been reported previously to render NK cells hyporesponsive, and hence these cells probably require a higher signal intensity to mount an effective response [27].

CFS/ME patients have significantly decreased extracellular-regulated kinase (ERK) 1/2 in CD56^{dim} CD16⁺ NK cells and significantly increased mitogen-activated protein kinase kinase (MEK) 1/2 and p38 in CD56^{bright} CD16^{dim/-} NK cells following incubation with K562 cells [28]. ERK 1/2 is activated in a PI3K-dependent manner that may also be associated with cytoplasmic Ca²⁺ ion levels through activation of TRPM3 [10]. In the absence of phosphatidylinositol biphosphate TRPM3 is not activated, resulting in reduced cytosolic Ca²⁺ [29]. Stimulating CD56^{dim} CD16⁺ NK cells with TG prior to PregS resulted in a significant elevation in cytotoxic activity.

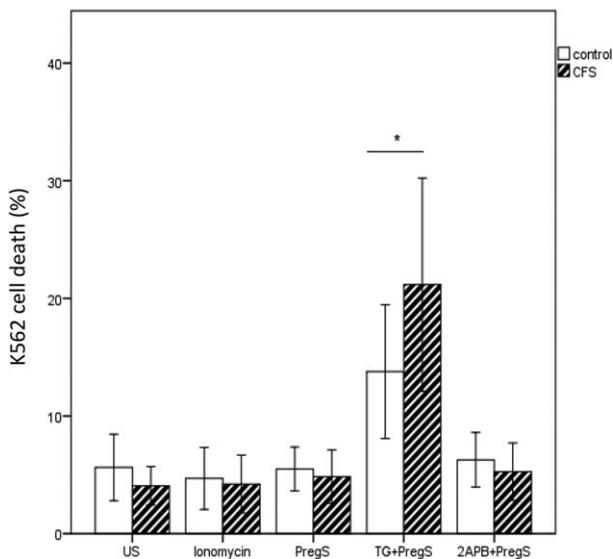


Fig. 5. Natural killer (NK) cell cytotoxic activity after incubation with ionomycin, PregS, TG + PregS and 2APB + PregS in HC and CFS/ME. Note significant elevation of K562 cell death in CFS/ME following TG + PregS. Data are represented as mean \pm standard error of the mean. Asterisk (*) represents statistical significance at $P < 0.05$. PregS = pregnenolone sulphate; 2-APB = 2-aminoethoxydiphenyl borate; TG = thapsigargin; CFS/ME = chronic fatigue syndrome/myalgic encephalomyelitis.

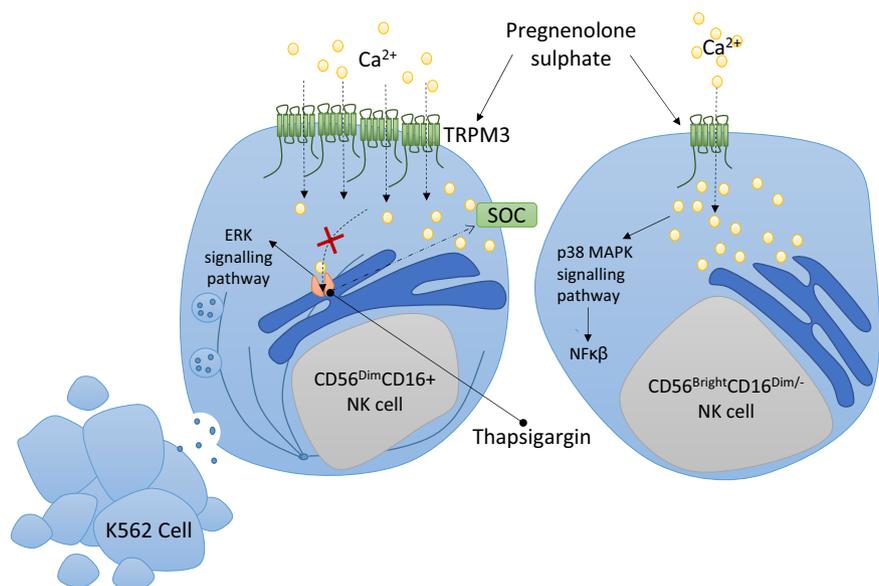
These findings are noteworthy, as it has been reported that depleting intracellular stores with TG increases activity of ERK. Phosphorylation of ERK 1/2 allows polarization of secretory granules for degranulation and therefore increased NK cell lysis in CFS patients [30–32].

CFS/ME patients have been reported to have a significant reduction in CD69 expression on T cells and NK cells upon stimulation, which may correspond to impaired

initial cell activation through a PKC-dependent signalling pathway [6]. However, this current investigation found no significant difference between groups. *In vitro*, degranulation is activated in NK cells when an immunosynapse is formed between NK cells and K562 tumour cells [7,33]. During degranulation, cytolytic granules containing lytic proteins are released and CD107a receptors are expressed on the NK cell surface. In the current investigation CD107a was not significantly different between groups, and this finding is supported by others [28]. PKC is activated by elevated intracellular Ca^{2+} concentration, which activates ERK signalling pathways and transcription factors [32,34,35]. Inhibition of PKC has been shown to impair signalling pathways that activate TRPM3-induced transcription factor activator protein-1 (AP-1) [35]. Our results also show that CD69 is reduced when NK cells are stimulated with TG. However, in combination with PregS stimulation, which facilitates an increase in cytoplasmic Ca^{2+} and activates PKC and ERK signalling pathways, NK cell cytotoxic activity in CFS/ME patients increased.

The current investigation confirms our previous reports of significant changes in TRPM3 receptor numbers in NK cells in CFS/ME. Following PregS stimulation, however, there was no significant increase in Ca^{2+} mobilization in CD56^{dim}CD16⁺ NK cells from CFS/ME patients. These novel findings suggest that TRPM3 receptor function is impaired, resulting in impaired Ca^{2+} mobilization with adverse consequences for homeostasis. As TRPM3 receptors are expressed throughout the human body, the current findings suggest that impaired TRPM3 function may play a significant role in the multisystemic pathomechanism of CFS/ME. Other TRP receptors, such as TRPM1, may possibly have a similar role in CFS/ME pathomechanism due to its sequence homology with TRPM3; however, further investigation is warranted [36]. We have previously identified

Fig. 6. A conceptual natural killer (NK) model for chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) patients. Pregnenolone sulphate (PregS) is a potent steroid that activated transient receptor potential melastatin 3 ion channels (TRPM3) on NK cell subsets. PregS in combination with thapsigargin elevated cytoplasmic calcium and is suggested to phosphorylate extracellular-regulated kinase (ERK) 1/2 and polarization of secretory granules for degranulation in CD56^{dim}CD16⁺ NK cells, resulting in target K562 cell death, whereas PregS-stimulated CD56^{bright}CD16^{dim/-} NK cells may activate p38 MAPK signalling pathways involved in the nuclear factor kappa B (NF- κ B) inflammatory immune response.



genomic changes in other TRPs and autonomic receptors, which should be investigated further [17,37,38].

The results of this study are considered preliminary, and are limited by our small sample size. Accordingly, we found that our CFS/ME patients were older compared with HC; however, routine pathological screening showed no difference between groups. As CFS/ME is a heterogeneous condition, future directions aim to optimize these findings in a larger cohort. In this pilot study, we characterized our CFS/ME patients by reduced NK cytotoxic activity compared with HC. We further aimed to capture those with significant functional impairment according to SF-36 and WHO DAS 2.0 disability scores. To define further the role of TRPM3 and Ca²⁺, future study will examine findings in distinct clinical phenotypes of CFS/ME to elucidate the distinct physiological effects of this mechanism.

Conclusions

TRPM3 activity and function in NK cells in CFS/ME patients is impaired, resulting in changes in Ca²⁺ ion concentration in the cytosol and intracellular stores which may, in turn, change the NK cells' activation threshold. Cytotoxic NK cells from CFS/ME patients may attempt to compensate for impaired TRPM3 receptors by increasing intracellular Ca²⁺ for sufficient NK cell activity. Increasing Ca²⁺ concentrations to activate the ERK signalling pathway may help to improve NK cell cytotoxicity in CFS/ME. Improvement of Ca²⁺-dependent NK activity may help to improve the immune system in CFS/ME patients to facilitate a quicker response to eliminate pathogens. Moreover, improvement of cell-to-cell interaction could improve the development of antigen-specific memory cells. This investigation may inform the pathomechanism of reduced NK cell cytotoxicity in CFS/ME patients.

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Disclosure

The authors declare that they have no disclosures.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site

Fig. S1. NK cell CD69 expression in HC and CFS/ME patients. A. CD56^{Bright}CD16^{Dim/-} NK cells expressed CD69 as cells/ml under various conditions including PregS, TG, 2APB and ionomycin. B. CD56^{Dim}CD16⁺ NK cell expressed TRPM3 as cells/ml under various conditions including PregS, TG, 2APB and ionomycin. Data are represented as Mean with SEM. Asterisk (*) represents statistical significance at $P < (0.05)$. Abbreviations: US: Unstimulated, PregS: Pregnenolone sulphate, TG: Thapsigargin, HC: Healthy controls, CFS/ME: Chronic Fatigue Syndrome/Myalgic Encephalomyelitis.