Exosome-associated mitochondrial DNA from patients with ME/CFS stimulates human cultured microglia to release IL-1β

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Research Article

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Abstract

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a debilitating disease that presents with fatigue, sleep disturbances, malaise and cognitive problems. The pathogenesis of ME/CFS is presently unknown and serum levels of potential biomarkers have been inconsistent. Here we show that serum mitochondrial DNA (mtDNA), associated with exosomes, is increased in ME/CFS only after exercise. Moreover, exosomes isolated from patients with ME/CFS stimulate significant release of IL-1β from cultured human microglia. These results provide evidence for a potential novel pathogenetic factor and target for treatment of ME/CFS.

Introduction

Myalgic encephalomyelitis/Chronic fatigue syndrome CFS (ME/CFS) is a chronic, debilitating disease affecting 1% of the US population [1] with an economic burden of about $20 billion.[2] The prevalence of ME/CFS is about 0.3% in the USA,[3] occurs predominantly in women, and is characterized by disabling fatigue. [5-9] However, the core symptoms of fatigue, sleep problems and cognitive difficulties exist across other comorbidities, such as fibromyalgia syndrome (FMS). {Wessely, 1999 27433 /id;Barsky, 1999 27434 /id;Ciccone, 2003 27432 /id; Natelson, 2019 27418 /id;Natelson, 2019 27420 /id}

The pathogenesis of ME/CFS is still unknown, but those with only ME/CFS showed more often difficulties in cognitive performance [14] and approximately twice as many ME/CFS patients developed their illness following a sudden, influenza-like onset compared to those with ME/CFS+FMS .[13] ME/CFS may be an autoimmune disease [16, 17] involving neuroinflammation. [18-21] Even though serum pro-inflammatory cytokine levels have been reported to be increased in ME/CFS patients [22] other studies using sedentary controls have not supported such findings either before or after stress induced by exercise or sleep deprivation. {27419;Natelson, 2019 27420 /id} As a result, it was suggested that ME/CFS may involve some dysfunction in the brain. [23] We proposed that local inflammation in the hypothalamus could dysregulate homeostasis. [24]

Extracellular vesicles (EVs) are membrane-bound vesicles ranging from 30-10,000 nm diameter, with exosomes considered the subfraction of 30-300 nm in diameter. [25] EVs have been shown to carry different types of cargo including microRNAs, proteins and lipids with the potential to alter pathophysiological processes. [26] In particular, exosomes have been implicated in brain disorders, [27,28] but their potential role in ME/CFS has not been adequately investigated.

Here we show that serum mitochondrial DNA (mtDNA), associated with exosomes, is increased only in ME/CFS patients as compared to both FMS and controls, and stimulates cultured human microglia to secrete IL-1b.

Methods

All patients with FMS fulfilled the American College of Rheumatology's 1990 criteria [4] and did not have comorbid ME/CFS. Serum from such patients was already in our possession from a previous study (Madrid, Spain). [29]

The normal controls were not related to any of the ME/CFS or FMS patient groups and were purchased from BBI Solutions (Cardiff, UK). Serum samples were labeled only with a code number, the age and gender of the subjects. All samples were processed immediately, and the serum was stored in –80°C until used for analysis.

Total EVs Isolation: Total EVs were isolated using the exoEasy Maxi Kit (Qiagen, Valencia, CA) from 1 mL of serum. Pre-filtered serum (0.8 µm syringe filter) were mixed with Buffer XBP and were bounded to an exoEasy membrane affinity spin column. The bound EVs were washed with Buffer XWP, were eluted with 400 µl Buffer XE (an aqueous buffer containing primarily inorganic salts) and were then ready to use for further analysis. The reason we used this commercialy available EVs purification kit is due to the limited amount of biological samples (2 mL serum from each ME/CFS patient and healthy control) available in our possession.

BCA assay: The concentration of total protein was quantified by the bicinchoninic acid (BCA) assay (Thermo Fisher Inc., Rockford, IL) using bovine serum albumin (BSA) as standard.

Electron microscopy: A drop of isolated serum-derived total EVs suspended in Buffer XE was deposited on Formvar-carbon-coated electron microscopy grids, fixed as above, immunolabelled and stained using the method as described before. All samples were analyzed at Harvard Medical School's Electron Microscopy (EM) Core Facility by Ms. Maria Ericsson, Manager of the Harvard Medical School EM Facility, using the Tecnai G2 Spirit BioTWIN transmission electron microscope (TEM).

Mitochondrial DNA: Total DNA was extracted from EVs using Qiagen DNA Micro extraction kit (Qiagen, CA). Mitochondrial specific DNA for 7S (mt-7S) was quantified by RT-qPCR using Taqman gene expression assays (Mt-7S: Hs02596861_s1; GAPDH: Hu, VIC, TAMRA, Applied Biosystems, Carlsbad, CA). Samples were run at 45 cycles using Applied Biosystems 7300 Real-Time PCR System. GAPDH DNA was used to exclude any genomic "contamination."

Cell culture: SV40 immortalized human adult microglia, frozen in the M1 pro-inflammatory state, were purchased from Applied Biological Materials Inc., (ABM, Vancouver, Canada). Microglia were cultured in Prigrow III medium (ABM) supplemented with 10 % FBS and 100 U/mL penicillin/streptomycin using BD PureCoat ECM Mimetic Cultureware Collagen I peptide plates (Thermo Sci.).

Cell viability assay: Cell viability was measured by Trypan blue (1%) exclusion.

Mediator assay: IL-1b was assayed using commercial ELISA kits from R&D Systems (Minneapolis, MN). Control cells were treated with equal volume of culture medium only.

Power analysis and statistics: Primary objective: Content of serum EV-mtDNA from ME/CFS and FMS patients. The required sample size to observe a difference of 30% in EV-associated mtDNA between ME/CFS patients and controls at 5% significance level with a power of 80% is at least 25 subjects/group. Since the variance between the two subgroups with and without FMS is not known, the number was increased to n=30 per group. Correlations between mtDNA and either subgroup were determined using the Spearman rank correlation test. Comparisons with control were done using either parametric t-test for independent samples or Mann-Whitney non-parametric test depending on normality of distribution to be checked with the Shapiro–Wilk's test. Comparisons between the groups were done with ANOVA and Wilcoxon post-hoc paired rank sum test.

Results

Serum levels of EV- associated mtDNA in ME/CFS and FMS patients.

EVs were isolated and characterized by electron microscopy confirming their spherical shape covered by a membrane (Fig 1A) and then by Western blot analysis. Total EV-associated protein was significantly lower in ME/CFS patients as compared to controls and was even lower after exercise (Fig. 1B).

The serum level of mtDNA (7S) was increased only after exercise in ME/CFS, and was not increased in FMS patients either before or after treatment with a dietary supplement (Fig. 2). Moreover, the ratio of mtDNA to that of EV-protein was significantly increased only in ME/CFS as compared to controls.

EVs can activate human microglia.

Total EVs isolated from patients with ME/CFS, but not FMS, stimulate significant release of IL-1β from cultured human microglia (Fig. 3). These results were obtained using mtDNA pooled from 5 subjects from either control, ME/CFS or FMS and not with mtDNA from each individual patient.

Discussion

We isolated total exosomes (30-100 nm fin diameter) from serum samples obtained from ME/CFS patients and controls as we reported previously. [30] Interestingly, we reported less total EV-associated protein obtained from *serum* of ME/CFS patients than controls unlike two other studies that reported no significant difference in EV yields obtained from *plasma* of ME/CFS patients and controls. [31, 32] In contrast, two other studies reported a significant increase in EV content in the *serum* of ME/CFS patients and controls. [33, 34] These apparent differences may depend on the activity status of the controls, use of serum or plasma, as well as method of EV isolation and purification, thus highlighting the need for standardization for future studies.

Exosomes are EVs with a size range of 30-100 nm in diameter and may carry different cargo than larger EVs (100-1000 nm in diameter). [25] EVs have been implicated in brain disorders,[27,28] but their role in ME/CFS had not been adequately investigated. EVs have been isolated from ME/CFS patients and

characterized especially for their content of microRNA. [32] Another study investigated the cytokine profile of plasma and MEs isolated from plasma of ME/CFS patients and reported no significant differences. [31] Hence, the amount of circulating cytokines whether free or EV=-associated may not be important, as compared to the ability of EVs to potentially stimulate the release of pro-inflammatory cytokines from microglia in the brain.

The source of exosomes in ME/CFS patients in not presently known. One possibility is that they derive from mast cells [35,36] either in the hypothalamus where they are particularly abundant [37, 38] perivascularly in close proximity to neurons [38, 39] or from peripheral mast cells and enter the brain by crossing the blood-brain barrier (BBB), which is known to be disrupted in neuropsychiatric disorders. [40,41] Mast cells and their mediators have been implicated in diseases comorbid with ME/CFS. [42] Moreover, mast cells are increased in the skin of patients with ME/CFS, [43, 44] who also show increased skin hypersensitivity. [45] Hyper-responsiveness of the bronchi, implying activation of mast cells, has also been noted in ME/CFS patients. [46] Activated mast cells could release additional mediators contributing to ME/CFS symptoms.[47, 48] The possible mast cell source of the EVs could be determined in the future by Western blot analysis for mast cell-specific surface markers (FceRI, MRGX2 and CD-117). [36]

Here we show that serum mitochondrial DNA (mtDNA), associated with exosomes, is increased only in ME/CFS patients as compared to both FMS and controls, and further increased after exercise in patients with ME/CFS. Moreover, we report that exosome-associated mtDNA stimulates cultured human microglia to secreteIL-1b. We had previously reported that cultured human mast cells stimulated by substance P secrete mtDNA. [49] Mitochondrial DNA could act as an “innate pathogen” leading to a localized auto-inflammatory response in the hypothalamus.[50-53] Analysis of the mitochondrial genome in ME/CFS cases indicated that individuals with a certain haplotype were more likely to exhibit certain neurologic symptoms, but there was no association with either susceptibility [54] or disease severity. [55] An unusual pattern of mtDNA deletions was reported in the skeletal muscle of one patient with ME/CFS. [56] Cellular bioenergetics has been reported to be impaired in patients with ME/CFS [57] and salivary mtDNA was found to be decreased in subjects with fatigue. [58] Even though mtDNA was shown to be neurotoxic in rat brain slices, [59, 60,61] the mtDNA molecular patterns N-formyl peptides and cardiolipin did not stimulate IL-6 or TNF release from HMC-3 microglia [62] implying that the entire mtDNA may be required for stimulation.

There are a number of limitations in this study. First, the number of subjects analyzed was small. Pathogenetic changes in ME/CFS patients may only occur in the brain, especially the hypothalamus, in which case we will have to await the availability of brain tissue to carry out analysis of the proposed biomarkers, as we recently reported for other CNS conditions. [63]

Conclusion

These results provide evidence for a novel pathogenetic factor involving exosome-associated mtDNA stimulating microglia release of IL-1b that could lead to the development of new treatments for ME/CFS.

Declarations

Ethics approval and consent to participate: Patients signed an informed consent approved by the appropriate Institutional Review Board to participate in this research.

Consent for publication: The authros conset to the publication of this manuscript.

Availability of data and materials: All data and materials are available for review.

Conpeting interests: The authors declare no conflict of interest.

Funding: ME/CFS Initiative Ramsay Award to TCT.

Authors contributions: ET performed the experiments and analyzed the results; TCT reviewed the experiments and the data, searched the literature, and wrote the manuscript; BN provided the serum and reviewed the manuscript.

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Compliance with Ethical Standards

Disclosure of potential conflicts of interest: N/A

Research involving Human Participants: HIRB approval obtained.

Informed consent: Signed.

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Figures

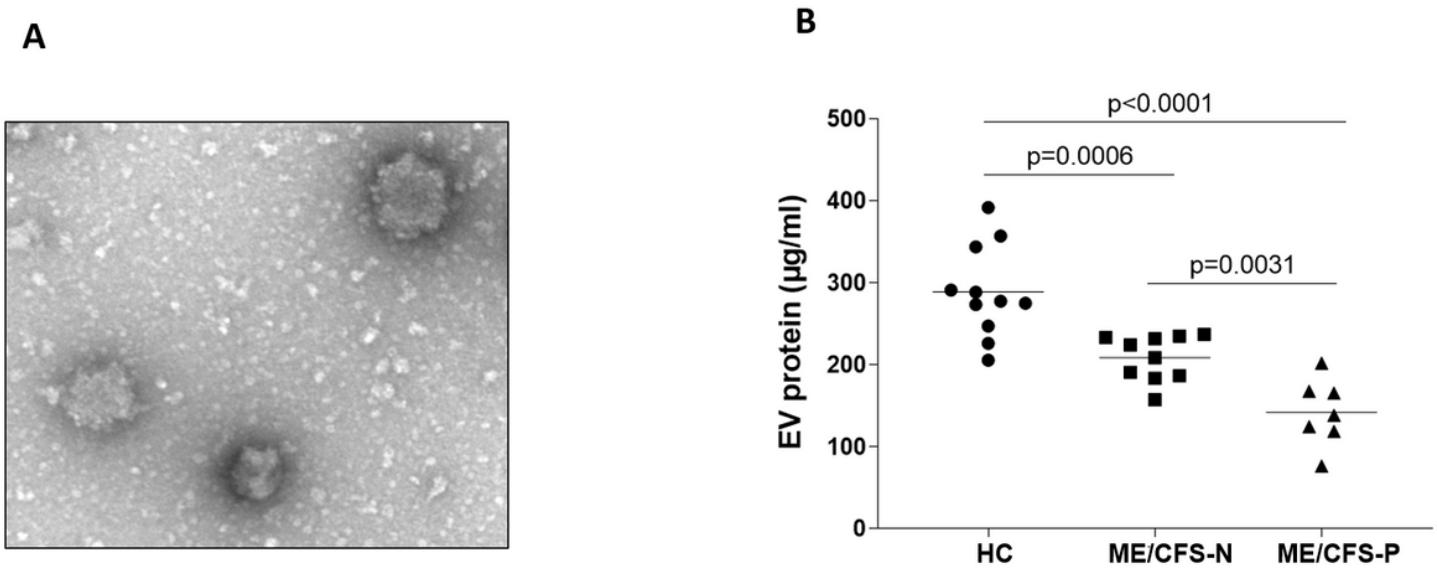

Figure 1

(A) Electron photomicrograph of EVs. (B) Total serum EV-associated protein in healthy controls (HC), ME/CFS patients without exercise (N) or past exercise (P), and FMS patients. Each dot represents individual subjects.

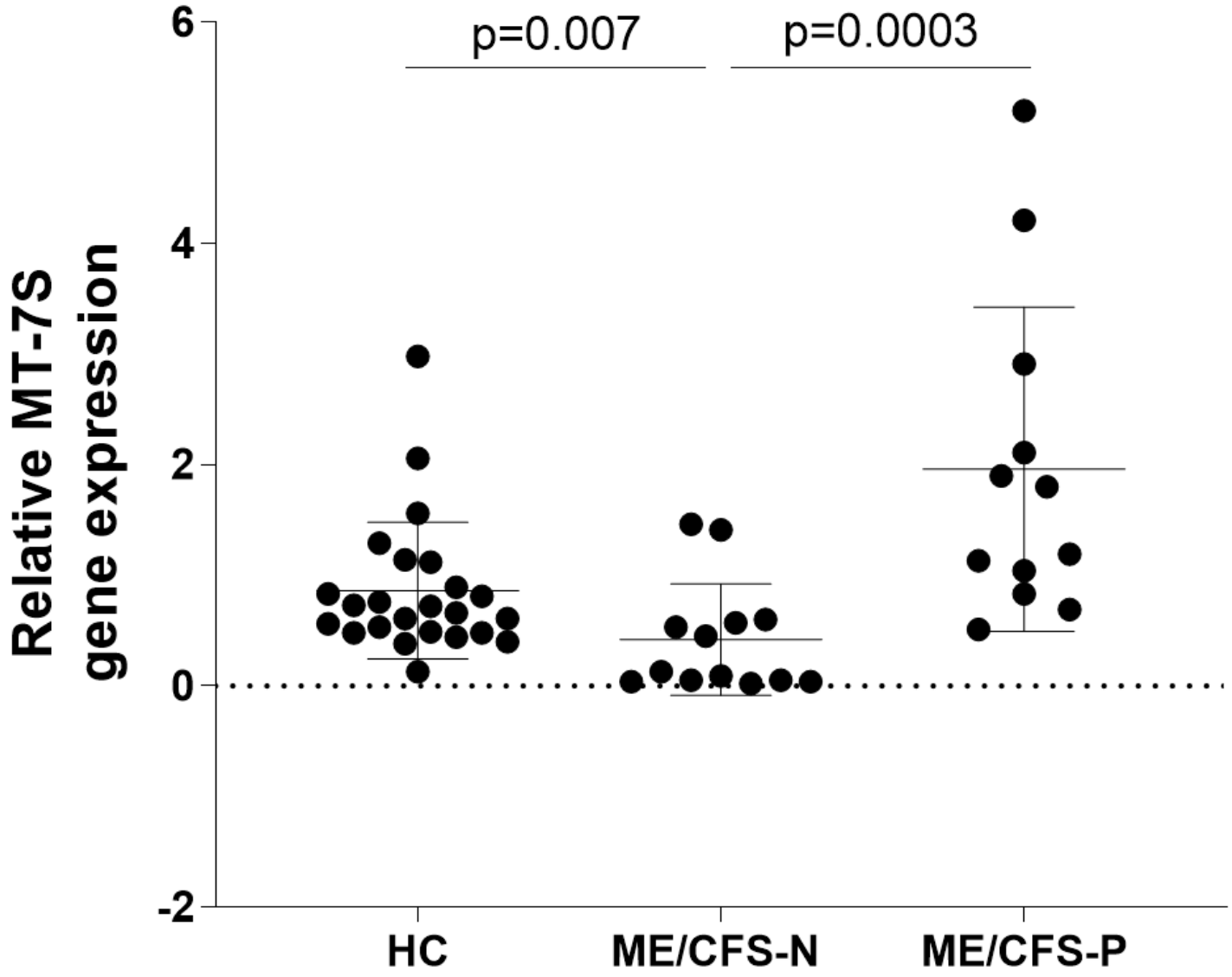

Figure 2

Gene expression of mtDNA(7S) in healthy controls (HC), ME/CFS patients without exercise (N) or past exercise (P), and FMS patients. Each dot represents individual subjects. mtDNA(7S) was normalized to the mean of all control samples. GAPDH was undetectable.

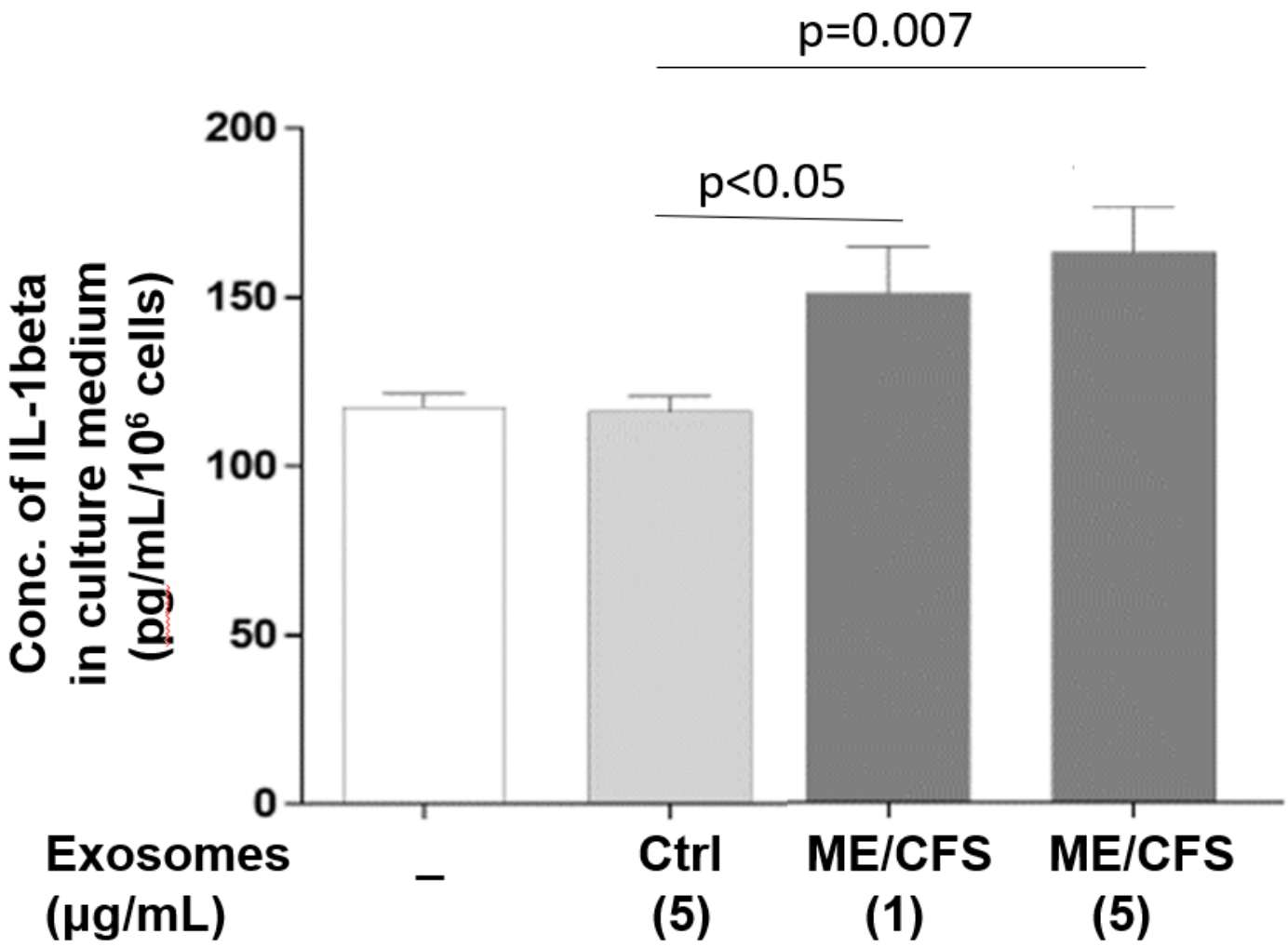

Figure 3

Effect of EVs on human microglia. Immortalized HM-SV40 microglia (1.0 x 105 cells) were stimulated with EVs (1 or 5 µg/mL as indicated) obtained and pooled from five subjects from each category (ctrl=control, ME/CFS or FMS). Secretion of IL-1β was measured in the supernatant fluid by ELISA (n=3, *p<0.05 and ****p<0.0001).