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Myalgic encephalomyelitis/chronic fatigue syndrome patients exhibit altered T cell metabolism and cytokine associations

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Abstract

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a complex disease with no known cause or mechanism. There is an increasing appreciation for the role of immune and metabolic dysfunction in the disease. ME/CFS has historically presented in outbreaks, often has a flu-like onset, and results in inflammatory symptoms. Patients suffer from severe fatigue and post-exertional malaise. There is little known about the metabolism of specific immune cells in ME/CFS patients. To investigate immune metabolism in ME/CFS, we isolated CD4+ and CD8+ T cells from 53 ME/CFS patients and 45 healthy controls. We analyzed glycolysis and mitochondrial respiration in resting and activated T cells, along with markers related to cellular metabolism, and plasma cytokines. We found that ME/CFS CD8+ T cells have reduced mitochondrial membrane potential compared to healthy controls. Both CD4+ and CD8+ T cells from ME/CFS patients had reduced glycolysis at rest, while CD8+ T cells also had reduced glycolysis following activation. ME/CFS patients had significant correlations between measures of T cell metabolism and plasma cytokine abundance that differed from healthy control subjects. Our data indicate that patients have impaired T cell metabolism consistent with ongoing immune alterations in ME/CFS that may illuminate the mechanism behind this disease.
Introduction

ME/CFS is a poorly understood and debilitating disease. An estimated 836,000-2.5 million Americans have ME/CFS (1). Patients suffer from severe fatigue, unrefreshing sleep, cognitive impairment, post-exertional malaise, pain, gastrointestinal symptoms, and orthostatic intolerance. In addition, many ME/CFS patients experience flu-like symptoms, such as tender or swollen lymph nodes, fever, muscular pain, headaches, and sore throats. Diagnosis is established through application of one or more sets of criteria, as summarized in a 2015 report from the Institute of Medicine (1). The disease is variable in severity, with approximately one-quarter of patients house- or bed-bound at some point in the course of their illness (1). The vast majority of patients with ME/CFS do not recover, and there are no known causes or approved treatments for ME/CFS (2).

Historically, there have been clustered outbreaks of ME/CFS worldwide, including major outbreaks in the 1980’s in the United States in Incline Village, NV and Lyndonville, NY (3). Onset of ME/CFS can be either sudden or gradual, but often resembles a flu-like illness (4). Some patients have diagnosed infections at the onset of the disease (2). Because of the outbreaks, onset of the illness, and symptoms, the potential identification of a pathogen in ME/CFS is a research priority. Despite this, no viral, bacterial or eukaryotic pathogen has yet been identified as causing ME/CFS.

Characterizing the immune system in patients is an alternative method to explore possible immune or infectious components in ME/CFS. There is substantial evidence that the immune system plays a role and is dysregulated in ME/CFS. Many studies have investigated the abundance of cytokines or immune cell subpopulations in ME/CFS.
patients, often with conflicting results. However, there have been changes identified in cytokines over the duration of the illness or between patients of differing severity (1, 5-7). Cytokines reported to be altered in abundance include TNFα, TGFβ, IFNγ, IL-1α, IL-1β, IL-6, and IL-4 (1, 5-10). Some studies have also reported differences in the frequency of CD4+ vs CD8+ T cells, regulatory T cells and/or memory T cells in ME/CFS patients (10-16).

Interestingly, Natural Killer (NK) cells from ME/CFS subjects have consistently been reported to exhibit reduced cytotoxicity compared to NK cells from healthy controls (1). There have similarly been reports of decreased CD8+ T cell cytotoxicity in ME/CFS patients, as well as decreased granzyme A and perforin in patient CD8+ T cells (16, 17). Others have reported increased dexamethasone sensitivity and decreased proliferation of CD4+ T cells, but no difference in cell death of either CD4+ or CD8+ T cells (10, 18). Overall, our understanding of functional impairment in ME/CFS T cells remains limited.

Due to the symptoms of fatigue and post-exertional malaise, there has also been substantial research into metabolism in ME/CFS patients. There is some evidence of oxidative stress and of decreased coenzyme Q10 in ME/CFS (19-22). There are conflicting findings regarding ME/CFS cellular mitochondrial content, but multiple studies have found no difference in specific mitochondrial complex activities (19, 23-25). Our laboratory previously reported no association between mitochondrial single nucleotide polymorphisms (SNPs) and ME/CFS, although some SNPs correlated with specific symptoms in patients (26). Others have also found no clinically relevant mitochondrial SNPs in ME/CFS patients (27, 28).
More recently, there have been multiple metabolomics analyses in plasma, serum or urine from ME/CFS patients compared to healthy controls (29-34). These have shown numerous differentially abundant metabolites in ME/CFS patients belonging to pathways such as fatty acid metabolism, the citric acid (TCA) cycle, glucose and amino acid metabolism (29-34). Alterations in plasma metabolites likely mean that cellular metabolism is also altered in ME/CFS.

Metabolism is critical to the function of all cells but is particularly imperative for the proper function of the immune system. Immunometabolism, or the study of how immune cell metabolism underlies the function and response of the immune system, plays a role in many human diseases. Specifically, quiescent T cells use little anabolic metabolism (35). In response to an immune challenge, T cells increase their utilization of oxidative phosphorylation, glycolysis, and glutaminolysis, while decreasing fatty acid oxidation (35-37). These shifts in metabolism require changes in the surface expression of substrate transporters (37). Changes in T cell metabolism are driven by specific signaling pathways and are highly regulated. Proper metabolic programs at rest and following an immune challenge are essential for optimal T cell effector function, proliferation, and viability (37, 38).

Dysfunction in immunometabolism can contribute to the establishment and/or maintenance of human disease. Increased T cell metabolism or reactive oxygen species (ROS) production can contribute to inflammation and/or autoimmunity (39-42). Conversely, hypometabolism, a failure to increase metabolism following an immune challenge, can help maintain a chronic infection or cancer progression (42-45).
Specifically, chronic infection can lead to T cell exhaustion, where metabolism and in turn effector functions are impaired (46, 47).

Characterizing immunometabolism in ME/CFS may provide insight into the mechanism of the disease, potential causes and targets for treatment. Mitochondrial dysfunction in neutrophils from individuals with ME/CFS has previously been reported (48, 49). A recent pilot study reported decreased glycolytic reserve in ME/CFS NK cells (50). Tomas et al. found reduced measurements of mitochondrial respiration in ME/CFS peripheral blood mononuclear cells (PBMCs) at rest, but no difference in glycolysis (51). However, we still do not know how the metabolism of other immune cells, such as T cells, is affected in ME/CFS. Additionally, the capacity of specific ME/CFS immune cells to reprogram their metabolism is unknown.

In order to investigate T cell metabolism and its potential role in ME/CFS, we sought to characterize metabolism in ME/CFS CD4+ and CD8+ T cells at rest and following activation. We collected samples from 45 healthy controls and 53 patients, from which we isolated both CD4+ and CD8+ T cells. We obtained extensive survey information on each subject. We then assayed both mitochondrial metabolism and glycolysis in resting and stimulated T cells. We also characterized mitochondria and GLUT1 abundance on these cells. Finally, we determined the abundance of cytokines in plasma from the same subjects and analyzed our data for correlations.
Results

Subjects in study population

Subjects were recruited at Simmaron Research in Incline Village, NV by Dr. Daniel Peterson, and fulfilled the Canadian Consensus Criteria. A total of 53 ME/CFS patients and 45 healthy controls were included in the study. The composition of the case and control groups was similar for gender and age (Table 1). Race was overwhelmingly Caucasian. Only one patient and one control identified as Hispanic or Latino. Patients had an average illness duration of 21.7 years, and most patients reported being ill for greater than 10 years (Table 1). In addition, patients reported an average of 6.7 years between their first symptoms and ME/CFS diagnosis (Table 1). Out of 53 ME/CFS patients, 24 reported a gradual onset and 26 reported sudden onset; onset type was unknown for the remaining 3 patients (Table 1). ME/CFS patients were also asked for the triggering event of their illness, if known. The vast majority of patients stated that either a known viral infection (n=18) or viral-like illness (n=23) preceded their illness (Table 1). We were unable to control for medications in this study, but all subjects were asked to provide a list of current medications and supplements.

All subjects were asked to complete the specific symptom severity form, which involves rating common ME/CFS symptoms from 0 if not experienced to 10 if very severe. Patients reported statistically significantly higher scores for all of the specific symptom severity scale items (Table 1). In particular, patients scored high on impaired memory or concentration, fatigue, muscle tenderness or pain, and post-exertional malaise (Table 1). Additionally, subjects completed the 36-Item Short Form Survey (SF-36), which calculates a score for various dimensions of health, with 100 indicating no
disability in a dimension and 0 indicating severe disability. ME/CFS patients had statistically significantly lower scores on all dimensions of the SF-36 survey, but especially in regard to physical health and vitality (Table 1). Patients reported an average Bell scale score of 37.1 compared to 96.7 in healthy controls (p<0.001) (Table 1). The Bell scale ranges from 0 to 100, where 100 reflects a healthy individual and 0 reflects severe disability or impairment (52). Thus, ME/CFS patient survey scores reflect substantial impairment compared to healthy controls and confirm our study population has expected characteristics of the disease.

Both ME/CFS and healthy control subjects were asked a series of questions about gastrointestinal conditions and/or symptoms, co-morbidities, and family health history. Thirteen ME/CFS patients had a previous cancer diagnosis, compared to 4 healthy controls (p=0.08) (Supplemental Table 1). Of 53 ME/CFS patients, 35 (66%) reported some kind of gastrointestinal symptom, while only 8 of 45 (17.8%) healthy controls reported gastrointestinal symptoms (p<0.001) (Supplemental Table 1).

Strikingly, 43.4% of patients reported being diagnosed with Irritable Bowel Syndrome, compared to only 6.7% of controls (p<0.001) (Supplemental Table 1). Thirty-three of 53 (62.3%) patients had at least one family member with an immune or inflammatory related disease, while only 15 of 45 (33.3%) healthy controls reported the same (p=0.008). This was largely driven by increased incidences of rheumatoid arthritis and type 1 diabetes for ME/CFS family members (Supplemental Table 1). No control subjects reported immune or inflammatory disease diagnoses, but 7 ME/CFS patients reported being diagnosed with at least one immune or inflammatory disease (p=0.03) (Supplemental Table 1). 73.6% of ME/CFS patients indicated having some kind of
allergy, compared to 48.9% of healthy controls (p=0.02) (Supplemental Table 1).

**CD4+ T cell mitochondrial metabolism is not altered in ME/CFS patients**

Blood samples were collected from ME/CFS and healthy control subjects at Simmaron Research in Incline Village, NV. Both patient and control samples were collected over approximately 18 months. PBMCs were isolated immediately, frozen, and later shipped overnight on dry ice to Cornell University. T cells were isolated from all samples using magnetic bead kits to separate CD8+ T cells by positive selection and CD4+ T cells by negative selection.

To investigate whether mitochondrial respiration is altered in patient and healthy control T cells, we used an Agilent Seahorse XFe96 extracellular flux analyzer, with a Mito Stress Test. The Mito Stress Test gives measurements of basal respiration, ATP production, maximal respiration, spare respiratory capacity, non-mitochondrial respiration and proton leak. In order to compare resting mitochondrial respiration, as well as the capability of patient T cells to remodel mitochondrial metabolism following activation, we also ran a Mito Stress Test after stimulation. For both CD8+ and CD4+ T cells, we assayed metabolism at rest and after overnight stimulation with anti-CD3/anti-CD28 beads and IL-2. We confirmed our activation method via flow cytometry analysis of the early activation marker CD69 on our cells (Supplemental Figure 1). Both ME/CFS and healthy control T cells had significantly increased CD69 mean fluorescent intensity (MFI) after overnight stimulation (Supplemental Figure 1). We did not always have enough T cells to include a subject in every assay. The number of viable PBMCs we received varied between different subjects and some subjects had a greater frequency
of T cells than others, as is expected (53). In particular, sample size for activated cell assays was reduced in order to preferentially assay cells in circulation.

For CD4+ T cells, there was no significant difference in basal mitochondrial respiration between healthy control and ME/CFS cells at rest (Figure 1A). There was a small, but non-significant decrease in maximal respiration in ME/CFS CD4+ T cells compared to healthy control CD4+ T cells, but not in spare respiratory capacity (Figure 1A). Both maximal respiration and spare respiratory capacity varied widely in healthy control and ME/CFS patient CD4+ T cells at rest (Figure 1A). There was no significant difference in proton leak between patient and control CD4+ T cells (Figure 1A). ATP production was slightly reduced in patient CD4+ T cells compared to healthy controls at rest, but there were two outliers (Figure 1A).

There was no significant difference in basal mitochondrial respiration between patient and healthy control CD4+ T cells after overnight stimulation (Figure 1B). There was also no difference in maximal respiration or ATP production (Figure 1B). There was a small increase in spare respiratory capacity and reduction in proton leak in ME/CFS CD4+ T cells compared to healthy controls, but neither difference was statistically significant (Figure 1B).

Both healthy control and ME/CFS CD4+ T cells displayed higher, although non-significant, basal and maximal mitochondrial respiration following activation compared to rest (Figure 1A-B). In both groups, there was substantial variation in metabolism after activation (Figure 1A-B). ATP production was significantly increased in healthy control CD4+ T cells following activation, and trended higher in ME/CFS CD4+ T cells (Figure 1A-B).
Overall, patient CD4+ T cells displayed no significant differences in mitochondrial respiration either at rest or after overnight stimulation. This suggests no major defects in mitochondrial metabolism within ME/CFS CD4+ T cells. Furthermore, ME/CFS CD4+ T cells do not appear to have a defect in their response to activation via mitochondrial respiration.

**ME/CFS CD8+ T cells have decreased proton leak and ATP production**

We then analyzed mitochondrial respiration in total CD8+ T cells. At rest, there was no significant difference in basal or maximal mitochondrial metabolism between healthy control and ME/CFS CD8+ T cells (Figure 1C). However, proton leak was significantly reduced in ME/CFS patients versus healthy controls, suggesting increased mitochondrial efficiency (Figure 1C). ATP production was slightly decreased, but there was high variability in both groups (Figure 1C). There was no difference in spare respiratory capacity between patient and control CD8+ T cells at rest (Figure 1).

After activation, there was a small reduction in basal mitochondrial respiration in ME/CFS CD8+ T cells compared to healthy controls (Figure 1D). ME/CFS CD8+ T cells also showed a small, but non-significant decrease in maximal respiration (Figure 1D). There was no difference in spare respiratory capacity between ME/CFS patient CD8+ T cells and healthy control cells after activation (Figure 1D). Proton leak trended lower in stimulated ME/CFS CD8+ T cells compared to healthy controls, consistent with the decrease in resting cells (Figure 1D). However, ATP production was significantly decreased in ME/CFS CD8+ T cells after activation, suggesting that the decreased proton leak does not result in improved ATP production (Figure 1D).
Healthy control CD8+ T cell metabolism showed a small increase in basal respiration from rest to activation (Figure 1C-D). Meanwhile, ME/CFS CD8+ T cells showed either no change or a decrease in basal mitochondrial metabolism after activation compared to resting cells (Figure 1C-D). The same pattern was found in both maximal respiration and ATP production (Figure 1C-D). Thus, CD8+ T cells may be less able to induce metabolism following activation.

Overall, these data indicate that there may be mitochondrial dysfunction in ME/CFS CD8+ T cells. The lack of increased metabolism in ME/CFS CD8+ T cells and additional differences between controls and patients after activation suggest an impaired ability to reprogram metabolism.

*CD4+ T cell mitochondrial mass and membrane potential does not differ between patients and controls*

To further investigate mitochondria in ME/CFS T cells, we sought to characterize mitochondrial morphology and membrane potential in T cells. Both CD4+ and CD8+ T cells were stained with MitoTracker Green (MTG) and MitoTracker Red (MTR) CMXRos simultaneously. MTG indicates mitochondrial mass, while MTR CMXRos is sensitive to mitochondrial membrane potential. Mitochondria were evaluated via confocal microscopy, as well as by flow cytometry. For confocal microscopy, cells were also stained with Hoechst 33342 to observe nuclei. Both microscopy and flow cytometry experiments were conducted after overnight rest or stimulation to indicate how patient and control T cell mitochondria respond to activation.
We did not observe a difference in either MTG or MTR CMXRos staining via confocal microscopy in CD4+ T cells (Figure 2A). Both at rest and following activation, the MitoTracker dyes co-stained well in merged images from patient and control CD4+ T cells (Figure 2A). We did not observe morphological differences in mitochondria within patient and control CD4+ T cells, although fluorescence image resolution is limited (Figure 2A).

Via flow cytometry, there was no significant difference in MTG MFI between healthy control and ME/CFS CD4+ T cells either at rest or after stimulation, indicating no difference in mitochondrial mass at either state (Figure 2B). There were also no significant differences in MTR CMXRos MFI between healthy control and ME/CFS patient CD4+ T cells at rest or after activation (Figure 2B). As such, there also does not appear to be a difference in mitochondrial membrane potential between patient and control CD4+ T cells.

**ME/CFS CD8+ T cells have decreased mitochondrial membrane potential**

We also visualized CD8+ T cells via confocal microscopy for mitochondrial morphology, mass and membrane potential. We did not observe a difference in mitochondria via MTG staining between healthy controls and ME/CFS patients (Figure 3A). However, at both rest and after stimulation, we observed ME/CFS CD8+ T cells with mitochondria that were stained by MTG but had little to no MTR CMXRos (Figure 3A). Meanwhile, CD8+ T cells from healthy controls showed even co-staining of MTG and MitoTracker CMXRos, similar to CD4+ T cells (Figure 3A). We did not see an observable difference in mitochondrial morphology between healthy control and
ME/CFS patient CD8+ T cells at either state. Further imaging experiments, particularly by electron microscopy, will be necessary to investigate whether there are differences not detectable by fluorescence microscopy.

We then used flow cytometry to quantify fluorescence of the two mitochondrial dyes in CD8+ T cells. Consistent with our microscopy images, there was no significant difference in MTG MFI between healthy control and ME/CFS patient CD8+ T cells at rest (Figure 3B). There was a small, but non-significant increase in MTG MFI following overnight stimulation in both control and patient cells (Figure 3B). MTR CMXRos was also slightly increased in healthy control and ME/CFS CD8+ T cells following activation. However, ME/CFS CD8+ T cells showed significantly lower MTR CMXRos both at rest and after activation compared to the healthy control CD8+ T cells (Figure 3). Thus, patient CD8+ T cells show no difference in mitochondrial mass at either state but do have decreased mitochondrial membrane potential compared to healthy control cells.

**ME/CFS CD4+ T cell glycolysis is reduced at rest compared to healthy control cells**

The second major, although less efficient, energy producing pathway in the cell is glycolysis. Glycolysis plays a significant role in immune cell function and is critical to T cell activation. We thus investigated whether glycolysis in ME/CFS T cells functions comparably to healthy control cells. Additionally, we determined whether ME/CFS T cells can properly stimulate glycolysis following activation. We assessed glycolysis in both CD8+ and CD4+ T cells at rest and after stimulation on the Seahorse XFe96 extracellular flux analyzer. For resting T cells, we analyzed glycolysis with a Seahorse Glycolytic Rate Assay, which provides quantitative measurements of basal glycolysis,
compensatory glycolysis and post-2DG acidification. Due to the limited cells we had available for assaying glycolysis after activation, we instead utilized a combined drug injection strategy that allowed simultaneous measurements of glycolysis and mitochondrial respiration. This involved a typical Mito Stress Test, with an added final injection of 2-DG and 5 measurements. Due to the use of this strategy, in stimulated cells we compared basal glycolysis, as well as “glycolytic capacity”, or the glycolysis induced by the injection of oligomycin, and post-2DG acidification. We did not make direct comparisons between compensatory glycolysis and glycolytic capacity, but we were able to compare basal glycolysis from rest to activation.

When we assayed glycolysis in CD4+ T cells at rest, basal glycolysis was significantly lower in ME/CFS patients versus healthy control cells (Figure 4A). Compensatory glycolysis was also significantly reduced at rest in ME/CFS CD4+ T cells (Figure 4A). Post-2DG acidification was slightly, but not significantly decreased in ME/CFS CD4+ T cells compared to healthy controls (Figure 4A). Interestingly, there was no difference in basal glycolysis rates between healthy control and patient CD4+ T cells after overnight stimulation, suggesting that activation is sufficient to overcome decreased resting glycolysis (Figure 4B). There was, however, substantial variation in glycolysis in stimulated ME/CFS cells. Likewise, glycolytic capacity was not significantly different between control and ME/CFS CD4+ T cells after activation, despite a small increase in patient cells (Figure 4B). Post-2DG acidification was significantly higher in ME/CFS CD4+ T cells compared to healthy controls, despite seeing a slight decrease in resting cells (Figure 4B). This may indicate increased extracellular acidification from
non-glycolytic sources in activated ME/CFS CD4+ T cells. Additionally, this may explain the small increase in glycolytic rates compared to control cells.

Between rest and stimulation, basal glycolysis increased in both healthy control and ME/CFS CD4+ T cells, but this difference was only significant in ME/CFS cells (Figure 4A-B). Post-2DG acidification was not different in healthy control CD4+ T cells from rest to activated but was significantly higher in patient CD4+ T cells after activation, suggesting increased extracellular sources of acidification following stimulation (Figure 4A-B).

As glycolysis depends on uptake of glucose through the main glucose transporter GLUT1, we also determined the surface abundance of GLUT1 via flow cytometry. The percent of GLUT1+ cells was significantly increased in healthy control subjects and had an increasing trend in ME/CFS patients after stimulation, when compared to resting cells. (Figure 4C). There were no significant differences in the percent of GLUT1+ cells between healthy control and ME/CFS CD4+ subjects either at rest or after activation (Figure 4C). However, there was a small increase in GLUT1+ cells in ME/CFS CD4+ T cells compared to controls at rest, despite the reduction in glycolysis.

**ME/CFS CD8+ T cell glycolysis is decreased at rest and after activation**

We then investigated glycolysis in CD8+ T cells. Similar to CD4+ T cells, basal glycolysis was significantly reduced in ME/CFS CD8+ T cells at rest (Figure 5A). Compensatory glycolysis was also significantly reduced in ME/CFS CD8+ T cells at rest (Figure 5A). Post-2DG acidification was reduced, although this difference was not significant. Unlike our CD4+ T cell results, ME/CFS CD8+ T cells also had significantly
lower basal glycolysis versus healthy control CD8+ T cells after activation (Figure 5B). There was no significant difference in glycolytic capacity or post-2DG acidification between ME/CFS and control stimulated CD8+ T cells (Figure 5B). There was high variability in glycolytic capacity in both groups (Figure 5B).

There were minimal changes in glycolysis between rest and activation for either healthy control or ME/CFS CD8+ T cells (Figure 5A-B). Interestingly, there was a slight, non-significant, increase in post-2DG acidification in ME/CFS CD8+ T cells following stimulation, similar to the response we observed in ME/CFS CD4+ T cells (Figure 5A-B). The reduced basal glycolysis after activation may indicate an impaired ability of CD8+ T cells to remodel glycolysis after activation, similar to what we observed in mitochondrial respiration.

We also quantified GLUT1+ cells via flow cytometry of ME/CFS and healthy control CD8+ T cells. Like CD4+ T cells, CD8+ T cells from both groups had an increased percentage of GLUT1+ cells following stimulation (Figure 5C). Additionally, there were again no significant differences in the abundance of GLUT1+ cells between healthy control and ME/CFS cells at either rest or after activation (Figure 5C). Nevertheless, there was a small decrease in GLUT1+ cells in patients compared to controls at rest (Figure 5C).

*Plasma cytokines uniquely correlate with T cell metabolism in ME/CFS patients*

In addition to PBMCs, we collected EDTA plasma from all subjects. To further investigate immune function in ME/CFS, we analyzed plasma cytokine abundance in 37 healthy control and 36 ME/CFS samples via a 48-plex magnetic bead-based
immunoassay. Out of the 48 cytokines/chemokines measured, 44 were detected. We compared cytokine abundances between ME/CFS patients and healthy controls. We did not find significant differences between ME/CFS patient and healthy control subjects for any of the 44 cytokines detected (Supplemental Table 2).

Although we did not see differences in plasma cytokine abundance between patients and controls, we were interested in determining whether T cell metabolism correlated with levels of plasma cytokines or survey data. To do this, we used a Spearman correlation test with a multiple testing correction via false discovery rate. We considered correlations with a FDR adjusted p-value (q-value) below 0.01 to be significant. We also analyzed correlations in patients and controls separately to see whether both groups shared the same relationships within the data. Finally, we determined whether T cell metabolism was different between patients with or without IBS or inflammatory bowel disease (IBD).

We did not find any significant correlations between T cell metabolism and survey data in either ME/CFS patients or healthy controls. Additionally, T cell metabolism was not significantly different in patients with IBS or IBD compared to those without either disease. However, we did observe a number of significant correlations between plasma cytokines and metabolism. Most interestingly, these correlations were unique in ME/CFS patients compared to the control group. In ME/CFS patients, we found significant negative correlations between resting basal glycolysis in CD8+ T cells and the abundance of IL-2, IL-8, IL-10, IL-12 p70, and SCGFβ (Figure 6 A-E). None of these correlations were significant in healthy control subjects (Figure 6A-E). Conversely, we found a significant positive correlation between resting basal glycolysis in CD8+ T
cells and IL-9 (Figure 6F). This same trend was present in healthy controls but was not significant (Figure 6F). Resting CD8+ T cell compensatory glycolysis had significant negative correlations with M-CSF and TNFα (Figure 6G-H). Interestingly, the opposite trends were observed in healthy controls (Figure 6G-H). Following stimulation, patient CD8+ T cell post-2DG acidification and glycolytic capacity both correlated negatively with M-CSF abundance (Figure 6I-J). In healthy controls, the same relationships existed, but were not significant (Figure 6I-J). As many of these cytokines are pro-inflammatory, it is surprising that they correlate with reduced metabolism in ME/CFS CD8+ T cells.

There were four significant correlations between cytokines and measures of metabolism in control subjects, primarily in activated CD4+ T cell data. Stimulated CD4+ T cell basal mitochondrial respiration and maximal respiration were positively correlated with proinflammatory cytokine IL-17 (Figure 7A-B). Meanwhile, in ME/CFS patients, these correlations were non-significant and maximal respiration was negatively associated with IL-17 (Figure 7A-B). There was a significant negative correlation between plasma IL-9 and activated CD4+ T cell basal respiration, which was not present in ME/CFS patients (Figure 7C). Finally, there was a significant negative correlation between resting CD8+ T cell spare mitochondrial respiratory capacity and G-CSF in healthy controls, but not in ME/CFS patients (Figure 7D). Overall, ME/CFS and healthy controls have unique correlations between plasma cytokines and T cell metabolism which further indicate immune alterations in ME/CFS.
Discussion

T cells have previously been implicated in ME/CFS through functional assays and surface marker characterization. The clustered outbreaks of ME/CFS, onset of disease and immune related symptoms all suggest an infectious trigger. Altered cytokine profiles, reduced NK cell cytotoxicity, and previously successful treatments for patients further implicate the immune system in the disease (1).

In our study population, we saw evidence of immune involvement in ME/CFS through our survey data. Of 53 ME/CFS patients, 41 reported either a known viral infection or a viral-like illness as the trigger of their illness. ME/CFS patients had a higher prevalence of gastrointestinal symptoms or disorders, which has previously been reported and linked to altered gut bacteria (54, 55). Furthermore, the increase in either co-morbid diagnosis or diagnoses of relatives with immune-related conditions in patients supports a role for the immune system and potential genetic predisposition for the disease.

Our study population was well balanced for sex, allowing us to characterize T cells in both males and females. Almost all subjects were Caucasian, which is common in ME/CFS studies but not necessarily reflective of the true patient population (1). Our population was comprised mainly of long duration patients, which is not surprising, given that the average time to diagnosis reported was nearly seven years. Thus, an analysis of short versus long duration patient T cell metabolism was not possible in this study, but a future investigation including more short duration patients may yield additional insight.
Based on the evidence of a dysfunctional immune system in ME/CFS patients and the increasing appreciation for the role immune metabolism plays in T cell function, we sought to investigate metabolism in ME/CFS CD4+ and CD8+ T cells. A previous study found reduced mitochondrial respiration in ME/CFS PBMCs compared to healthy controls, but our study is the first investigation of specific T cell metabolism in ME/CFS (51). We characterized the two main energy producing pathways, oxidative phosphorylation and glycolysis, in cells and their underlying cellular components.

Our study of mitochondrial respiration found no dysfunction in mitochondrial metabolism in ME/CFS CD4+ T cells, nor any difficulty in increasing respiration in response to activation. The wide variation in many measures of metabolism was expected, but limited differentiation between patient and control cells. Our main limitation was sample size, primarily for overnight stimulation experiments. As expected and previously shown, both ME/CFS and healthy control CD4+ T cells increased mitochondrial respiration after activation (37, 56). There were also no differences in mitochondrial mass or membrane potential between ME/CFS patient and healthy control CD4+ T cells. Surprisingly, there was no significant increase in mitochondrial mass following activation, which has previously been demonstrated (57, 58). This may relate to the stimulation methods used. It is worth noting that MitoTracker MFI also ranged greatly in all populations.

In CD8+ T cells, we observed only a significant reduction in proton leak at rest in ME/CFS cells compared to healthy controls. Decreased proton leak would suggest increased mitochondrial respiratory efficiency, yet basal respiration, maximal respiration and ATP production were not significantly different in ME/CFS CD8+ T cells compared
to healthy controls. After activation, we observed a similar phenotype. As a whole, ME/CFS CD8+ T cells did not increase basal respiration to the same extent as healthy control cells following stimulation, suggesting an impairment in their ability to undergo metabolic reprogramming.

Our investigation of mitochondria in CD8+ T cells through imaging revealed a significant reduction in mitochondrial membrane potential in ME/CFS CD8+ T cells at both rest and after activation. This was surprising in light of the observed decrease in proton leak in patient cells, but is nonetheless supported by other observations of hypometabolism in ME/CFS immune cells (50, 51, 59). Furthermore, a subset of ME/CFS CD8+ T cell samples had more mitochondrial mass than healthy control subjects at rest. Both healthy control and ME/CFS CD8+ T cells had expected increases in mitochondrial mass and membrane potential after activation, although this difference was non-significant and not sufficient to overcome the reduced mitochondrial membrane potential in ME/CFS cells (60). In fact, the difference in mitochondrial membrane potential was greater following overnight stimulation. This may help explain an impaired metabolic response to activation and greater observable differences in mitochondrial respiration in stimulated cells. Decreased mitochondrial membrane potential has been seen following chronic viral infection, and is a common feature of T cell exhaustion (43, 45, 61).

In both CD4+ and CD8+ T cells from ME/CFS patients, we found significant reductions in basal glycolysis and compensatory glycolysis at rest. Interestingly, reduced plasma glucose in ME/CFS has previously been reported (34). Reductions in glucose metabolism, without concurrent increases in mitochondrial respiration, indicate
hypometabolism in ME/CFS cells. Following stimulation, there was no significant difference in CD4+ T cell basal glycolysis or glycolytic capacity, indicating that activation is sufficient to overcome resting defects in glycolysis. ME/CFS and healthy control CD4+ T cells both increased glycolysis from rest to activation as expected, again suggesting that ME/CFS CD4+ T cells are capable of proper metabolic reprogramming (36). In contrast, ME/CFS CD8+ T cells still had significantly decreased basal glycolysis compared to healthy controls after activation. Along with our findings in CD8+ T cell mitochondrial respiration after activation, this supports an impairment in the ME/CFS CD8+ T cell metabolic response to activation. Nevertheless, there were minimal changes in rates of glycolysis in CD8+ T cells from patients or healthy controls between resting and activated states.

In both CD4+ and CD8+ T cells, the percentage of GLUT1+ cells was increased following stimulation, as previously shown in T cells (62). There were no significant differences between patients and controls. There was a slight increase in the percentage of ME/CFS CD4+ GLUT1+ cells compared to controls. However, there was a small reduction in the percentage of ME/CFS CD8+ T cell GLUT1+ cells at rest compared to controls, which is consistent with the decreased in glycolysis at rest. A previous study of T cell exhaustion demonstrated increased, rather than decreased, GLUT1 with impaired glycolysis (61). Additionally, glucose may be used for alternative pathways which are not measured by the Seahorse assays.

When we analyzed all of our data for correlations, we found significant correlations between plasma cytokine abundance and measures of T cell metabolism, which were unique in ME/CFS patients compared to controls. ME/CFS CD8+ T cell
glycolysis had multiple significant correlations with cytokines that are known to be proinflammatory or growth factors (63-65). This is especially interesting given the reduced basal glycolysis we observed in ME/CFS patients. In particular, IL-2 would be expected to induce glycolysis in T cells, rather than impair glycolysis (66).

Proinflammatory cytokines might be expected to be positively correlated with T cell metabolism, yet they were negatively correlated with ME/CFS patient T cell metabolism. In healthy controls, the proinflammatory cytokine IL-17 was positively correlated with stimulated CD4+ T cell basal and maximal respiration (63). Interestingly, these same relationships had negative trends in ME/CFS patients.

Other cytokines of interest had unique relationships with ME/CFS and healthy control T cell metabolism. IL-10, which was also negatively correlated with ME/CFS CD8+ T cell glycolysis, is an immunosuppressive cytokine that has previously been linked to chronic infection, immune cell exhaustion and inhibition of T cell activation (46, 63, 67). In these examples, diminished T cell metabolism would be predicted so that the negative correlation with glycolysis in ME/CFS CD8+ T cells is expected. Nonetheless, the absence of this correlation in healthy controls is notable. IL-9, which was positively correlated with ME/CFS CD8+ T cell glycolysis, is a T cell growth factor but has complex effects (63). In breast cancer patients, IL-9 producing Th9 cells led to improved CD8+ T cell cytotoxicity, which would require an increase in cellular metabolism (68). IL-9 was negatively correlated with activated CD4+ T cell basal respiration in healthy controls, which could be related to IL-9 improving regulatory T cell function (69).

Cytokines produced by other immune cells or T cell subsets are also secreted into plasma and contributed to our measurements. Nevertheless, ME/CFS CD8+ T cells
have multiple negative correlations between metabolism and plasma cytokines where positive correlations might be expected (63). This suggests that the relationship between proinflammatory cytokines in plasma and T cell metabolism is altered in ME/CFS patients and implicates specific cytokines for further investigation in ME/CFS.

It is clear that the immune system plays a role in ME/CFS. Our data indicate that there are existing reductions in resting T cell metabolism in patients. In particular, CD8+ T cells have altered mitochondrial membrane potential and an impairment in their metabolic response to activation. Both CD4+ and CD8+ T cells have significant reductions in glycolysis. This hypometabolism in T cells aligns with other findings of hypometabolism in ME/CFS cells (50, 51, 59). Furthermore, ME/CFS patients appear to have altered relationships between plasma cytokine abundance and T cell metabolism, where proinflammatory cytokines unexpectedly correlate with hypometabolism. Such a dysregulation may indicate that ME/CFS T cells have lost responsiveness to some proinflammatory cytokines. Along with hypometabolism in immune cells, this is consistent with a possible ongoing infection (42), though such an agent has not yet been identified. A high priority moving forward will be determining the mechanism behind hypometabolism in ME/CFS T cells, as well as how altered metabolism affects the function of these cells.
Methods

Study Population

Study participants were recruited by Simmaron Research in Incline Village, Nevada. ME/CFS patients were established patients of Dr. Daniel Peterson. Healthy controls and ME/CFS patients completed approved questionnaires including an SF-36, Bell Scale, specific symptom inventory and additional questions regarding symptoms, co-morbidities and family health history.

Sample collection and processing

Eighty mL of blood were collected from each subject in EDTA tubes. Blood was immediately processed to collect aliquots of whole blood, plasma and PBMCs. Briefly, EDTA tubes were spun at 500 xg for 5 minutes and plasma was removed and stored at -80°C. Blood was diluted 1:2 in PBS and layered over Histopaque 1077 in 50 mL SepMate tubes. SepMate tubes were spun at 1200 xg for 10 minutes. Excess plasma was removed and cells were poured into a clean 50 mL conical tube. Cells were washed once with PBS at 120 xg to remove platelets, then a second time at 300 xg for 5 minutes. PBMCs were then resuspended in freezing medium (60% RPMI 1640, 30% FBS, 10% DMSO) and stored in isopropanol containing freezing containers at -80°C to slow freezing. PBMCs and plasma aliquots were shipped overnight on dry ice within one month of isolation to Cornell University. PBMCs were stored long term in liquid nitrogen, while plasma was stored at -80°C.
**Immune cell isolation**

Specific immune cell subsets were isolated via STEMCELL EasySep kits on an EasyEights magnet. PBMCs were thawed quickly in a 37°C water bath. Following a wash in RPMI 1640, PBMCs were treated at room temperature with 10 mg/ml DNase I for 10 minutes, then strained through a 37um cell strainer to remove any remaining clumps. After a second wash, cells were isolated using the EasySep Human CD19 Positive Selection II Kit, EasySep Human CD56 Positive Selection II Kit, EasySep Human CD8 Positive Selection II Kit, and EasySep Human CD4+ T Cell Isolation Kit, sequentially, and following manufacturer’s instructions. Isolated cells were frozen and stored as described above.

**Extracellular flux analysis**

Extracellular flux analysis experiments were conducted at Dartmouth-Hitchcock Medical Center or at the Cornell University Biotechnology Resource Center on a Seahorse XFe96. Cells analyzed at Dartmouth-Hitchcock Medical Center were transported on dry ice and were immediately placed in a -150°C freezer. Cells for resting assays were thawed on the day of the experiment and washed in Seahorse assay media (RPMI without phenol red, 10mM glucose, 1mM HEPES, 2 mM glutamine, 10 mM sodium pyruvate). Cells were counted on a Bio-Rad TC20 with trypan blue to assess viability prior to seeding in triplicate in a Seahorse XFe96 Cell Culture Microplate treated with Cell Tak (22.4 mg/mL). The plate was spun at 300 xg for 1 min with no brake to adhere cells. Assays were run using the Seahorse Mito Stress kit, Seahorse Glycolytic Rate Assay, or a Mito Stress kit with an added final injection of 2-DG with 5
measurements. Drugs were injected at the following final concentrations: 1 uM oligomycin, 1 uM FCCP, 0.5uM rotenone/antimycin A, and 50 mM 2-DG. For activation assays, cells were cultured in complete RPMI overnight prior to the assay, plus stimulating reagents 25uL/mL STEMCELL Immunocult (anti-CD3 and anti-CD28), plus 80U/mL IL-2 (STEMCELL Technologies).

Seahorse data were first normalized in Wave Software per 100,000 viable cells based on the cell seeding density. Data was further analyzed in RStudio. All Seahorse variables were calculated according to Agilent Seahorse report generator manuals. Glycolytic capacity, determined for our activated cell assays, was determined to be the maximum glycolytic proton efflux rate after injection of oligomycin. For glycolysis data, glycolytic specific proton efflux rate was calculated as described by Agilent Technologies (70). Data were only retained if at least two replicates gave quality data for a given variable. Basal respiration and maximal respiration were required to be positive (above non-mitochondrial respiration levels), while ATP production could not be greater than total basal respiration. Basal glycolysis was required to be higher than post-2DG acidification. Seahorse variable measurements are thus an average of 2-3 technical replicates.

**Flow cytometry**

Flow cytometry analysis was done on the BD FACS Aria II in 5mL round bottom polystyrene tubes or on the Thermo Fisher Attune NxT Analyzer with a 96 well deep well round bottom plate at the Cornell Biotechnology Resource Center. Each cell type was stained with a specific cocktail of antibodies at rest and following stimulation.
(Supplemental Table 3). Cells were stimulated as described above. All cells were thawed and cultured overnight in complete RPMI 1640 prior to staining. Flow cytometry analysis was conducted immediately following the staining protocol. Data analysis was conducted using Kaluza (Beckman Coulter). For CD8+ T cells, cells were first gated as viable, then CD3+CD8+ were gated for further analysis. The percent of GLUT1+ cells and the MFI of MTG, MTR CMXRos, and CD69 were calculated in this population. For CD4+ T cells, CD4+ or CD3+CD4+ were gated following live/dead exclusion. Then, the percent of GLUT1+ cells and the MFI of MTG, MTR CMXRos, and CD69 were determined.

Confocal microscopy

Confocal microscopy imaging of immune cells was completed at the Cornell Biotechnology Resource center on a Zeiss LSM710. Cells were incubated at 37°C overnight, with or without stimulating reagents, as described above. Cells were stained for 30 minutes at 37°C with Hoechst 33342 (2ug/mL), MitoTracker Green (100nM), and MitoTracker Red CMXRos (50nM). Cells were then washed in RPMI 1640 media without FBS and moved to a glass-bottom 24-well MatTek plate coated with 22.4 mg/mL of Cell-Tak. The plate was spun down at 300 xg for 1 minute with no brake. Images were taken immediately following staining using a 63x oil immersion objective with 12-bit depth, bi-directional scanning, and a 3.6x zoom of the area of interest. Images were captured either as a single image or by focus stacking. Maximum intensity projections were created from the focus stacks using Zen 2.5 software (Zeiss).
Plasma cytokine analysis

EDTA plasma was thawed and diluted 1:2 prior to analysis. Forty-eight cytokines and chemokines were measured simultaneously for each participant’s plasma sample using the Bio-Plex Pro Human Cytokine Screening Panel, 48-plex (Bio-Rad, Hercules, CA, USA). Samples were run in duplicate on a MAGPIX® Multiplexing System (Luminex) at the Human Nutritional Chemistry Service Laboratory at Cornell University. The kit is designed to measure the following: IL-1 superfamily: IL-1α, IL-1β, IL-1Ra; IL-2, IL-2Rα, IL-3, IL-4, IL-5, IL-7, IL-9, IL-13, IL-15, IL-18; IL-6 family: IL-6, leukemia inhibitory factor (LIF); IL-12 family: IL-12p40, IL-12p70; IL-10; IL-17; Interferon family: IFNα2, IFNγ; Tumor Necrosis Factors: TNFα, TNFβ, TNF-related apoptosis-inducing ligand (TRAIL); granulocyte-macrophage colony-stimulating factor (GM-CSF); CC chemokines: monocyte chemoattractant protein-1 MCP-1 (CCL2), macrophage inflammatory protein 1-alpha/beta MIP-1α (CCL3), MIP-1β (CCL4), regulated upon activation, normal T cell expressed, and secreted RANTES (CCL5), monocyte chemotactic protein-3 MCP3 (CCL7), eotaxin (CCL11), cutaneous T-cell-attracting chemokine CTACK (CCL27); CXC chemokines: growth regulated protein alpha GROα (CXCL1), IL-8 (CXCL8), monokine induced by IFNγ MIG (CXCL9), IP-10 (CXCL10), stromal cell-derived factor 1-alpha SDF-1α (CXCL12); PDGF family/VEGF subfamily: platelet-derived growth factor PDGFBB, vascular endothelial growth factor VEGFA; and macrophage migration inhibitory factor (MIF), lymphocyte chemoattractant factor IL-16 (LCF), stem cell growth factor-beta (SCGF-β), fibroblast growth factor basic (FGFb), beta-nerve growth factor (βNGF), hepatocyte growth factor (HGF) , stem cell factor
(SCF), macrophage colony-stimulating factor (M-CSF), and granulocyte colony-stimulating factor (G-CSF). Concentrations were calculated using the software xPONENT® 4.2 for MAGPIX® (Luminex). A five-parameter logistic regression model (5PL) with weighting was used to create standard curves derived from the known reference concentrations supplied by the manufacturer and to calculate final concentrations (expressed in pg/ml) by interpolation. This method gives the greatest dynamic range for each standard curve (71). For statistical comparisons, samples with concentrations below the limit of detection (LOD) were assigned the value corresponding to the mid-point between zero and the LOD for that analyte.

Statistics
Statistical analysis was conducted in RStudio and Microsoft Excel. For survey data, pairwise statistical testing was done via Wilcoxon rank-sum test, while dichotomous response questions were analyzed by Chi-squared test. For all experiments, pairwise comparisons between control and ME/CFS cells were made via a Wilcoxon rank-sum test. For plasma cytokine analysis, a Wilcoxon rank-sum test was used for group comparison and p-values were adjusted using the false discovery rate for multiple testing correction. For comparison of four groups (ME/CFS rest, Controls rest, ME/CFS activated, Controls activated), statistical testing was conducted via a Kruskal-Wallis test followed by a Dunn’s test with multiple testing correction via false discovery rate. For correlation testing, Spearman correlation tests were conducted on all data, followed by a multiple testing correction via false discovery rate. Q-values less than 0.01 were considered significant.
Study approval

Human subject protocols were approved by the Western Institutional Review Board (# 20161887). All subjects gave written informed consent.

Author Contributions:
Designed research (AHM, MRH, JM, LG); conducted experiments (AHM, JM, LG); analysis of data (AHM, JM, LG, MRH); manuscript writing (AHM, MRH, JM, LG); diagnosis of subjects (DLP); clinical coordination (MM, CGG).

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BioRender.com. Finally, we would like to thank the many ME/CFS and control subjects who generously participated in this study.
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Mitochondrial respiration parameters in healthy control and ME/CFS CD4+ T cells after overnight stimulation with anti-CD3/anti-CD28 and IL-2, including non-mitochondrial respiration (n=12/11), basal respiration (n=12/11), maximal respiration (n=11/11), proton leak (n=7/7), ATP production (n=7/7) and spare respiratory capacity (n=11/11). Boxplots represent median (middle line), 25th and 75th quartiles (bottom and top edges of box), whiskers (1.5x interquartile range) and outliers are defined as values beyond whiskers. *P < 0.05, **P < 0.01, and ***P < 0.001, by Wilcoxon rank-sum test.
Figure 2. Mitochondrial mass and membrane potential do not differ between healthy control and ME/CFS CD4+ T cells. (A) MitoTracker green, MitoTracker Red CMXRos and Hoechst 33342 staining of representative resting and activated control and ME/CFS CD4+ T cells. The experiment was conducted 4 times for each condition (scale bar 5um). (B) MitoTracker Green and MitoTracker Red CMXRos Mean Fluorescence Intensity as determined by flow cytometry in healthy control and ME/CFS CD4+ T cells at rest and after overnight activation (n=15/14/17/16). Error bars represent mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001, by Kruskal-Wallis followed by Dunn’s test with multiple testing correction (false discovery rate).
Figure 3. Mitochondrial membrane potential is decreased in ME/CFS patient CD8+ T cells. (A) MitoTracker green, Mitotracker Red CMXRos and Hoechst 33342 staining of representative resting and stimulated control and ME/CFS CD8+ T cells. The experiment was conducted 4 times for each condition (scale bar 5um). (B) Mitotracker Green and Mitotracker Red CMXRos Mean Fluorescence Intensity as determined by flow cytometry in healthy control and ME/CFS CD8+ T cells at rest and after overnight activation (n=15/15/17/14). Error bars represent mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001, by Kruskal-Wallis followed by Dunn’s test with multiple testing correction (false discovery rate).
Figure 4. Basal glycolysis is reduced in ME/CFS CD4+ T cells. (A) Resting glycolysis measurements from Seahorse extracellular flux analysis in ME/CFS and healthy control CD4+ T cells, including basal glycolysis (n=28), compensatory glycolysis (n=15/16), and post-2DG acidification (n=22/17). (B) Glycolysis measurements in stimulated ME/CFS and healthy control CD4+ T cells, including basal glycolysis (n=10/11), glycolytic capacity (n=10/11), and post-2DG acidification (n=7/10). (C) Percent GLUT1+ cells in resting and activated CD4+ T cells from ME/CFS patients and healthy controls (n=14/14/16/13). Boxplots represent median (middle line), 25th and 75th quartiles (bottom and top edges of box), whiskers (1.5x interquartile range) and outliers are defined as values beyond whiskers. For dot plots, error bars represent mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001, by Wilcoxon rank-sum test (A and B) and Kruskal-Wallis followed by Dunn’s test with multiple testing correction (false discovery rate) (C).
Figure 5. Basal glycolysis is reduced in ME/CFS CD8+ T cells. (A) Resting glycolysis measurements from Seahorse extracellular flux analysis in ME/CFS and healthy control CD8+ T cells, including basal glycolysis (n=21/20), compensatory glycolysis (n=13/12), and post-2DG acidification (n=20/18). (B) Glycolysis measurements in stimulated ME/CFS and healthy control CD8+ T cells., including basal glycolysis (n=14/11), glycolytic capacity (n=14/11), and post-2DG acidification (n=13/9). (C) Percent of GLUT1+ cells in resting and activated CD8+ T cells from ME/CFS patients and healthy controls (n=14/13/14/14). Boxplots represent median (middle line), 25th and 75th quartiles (bottom and top edges of box), whiskers (1.5x interquartile range) and outliers are defined as values beyond whiskers. For dot plots, error bars represent mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001, by Wilcoxon rank-sum test (A and B) and Kruskal-Wallis followed by Dunn’s test with multiple testing correction (false discovery rate) (C).
Figure 6. Plasma cytokines are uniquely correlated with T cell metabolism in ME/CFS patients. Significant correlations between plasma cytokines and cellular metabolism in patients and non-significant correlations in healthy controls. Correlations between resting CD8+ T cell basal glycolysis and (A) IL-2 (n=19 patients/21 controls), (B) IL-8 (n=18 patients/19 controls), (C) IL-10 (n=19 patients/21 controls), (D) IL-12 p70 (n=19 patients/21 controls), (E) SCGFβ (n=19 patients/20 controls), (F) and IL-9 (n=19 patients/21 controls), between resting CD8+ T cell compensatory glycolysis and (G) M-CSF (n=12 patients/12 controls), (H) and TNFα (n=12 patients/12 controls), (I) between activated CD8+ T post-2DG acidification and M-CSF (n=9 patients/13 controls), (J) between activated CD8+ T cell glycolytic capacity and M-CSF (n=10 patients/14 controls). All correlations tested by Spearman correlation test with multiple testing correction (false discovery rate), with q<0.01 considered significant.
Figure 7. Plasma cytokines correlate with T cell metabolism in healthy controls. Significant correlations between plasma cytokines and cellular metabolism in healthy control subjects and non-significant correlations in patients. Correlations between (A) activated CD4+ T cell basal respiration and IL-17 in controls (n=12) and patients (n=10) (B) between activated CD4+ T cell maximal respiration and IL-17 in controls (n=15) and patients (n=10), (C) between activated CD4+ T cell basal respiration and IL-9 in controls (n=12) and patients (n=10), (D) between resting CD8+ T cell spare respiratory capacity and G-CSF in controls (n=19) and patients (n=20). All correlations tested by Spearman correlation test with multiple testing correction (false discovery rate), where q<0.01 is considered significant.
Table 1. Study population characteristics and survey response

<table>
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<tr>
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<th>ME/CFS (n=53)</th>
<th>Healthy Controls (n=45)</th>
<th>p-value</th>
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<td>Impaired memory or concentration</td>
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<td>52 ± 8.2</td>
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<td>Mental Component Score</td>
<td>43.8 ± 11</td>
<td>53.7 ± 10.4</td>
<td>p&lt;0.001</td>
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