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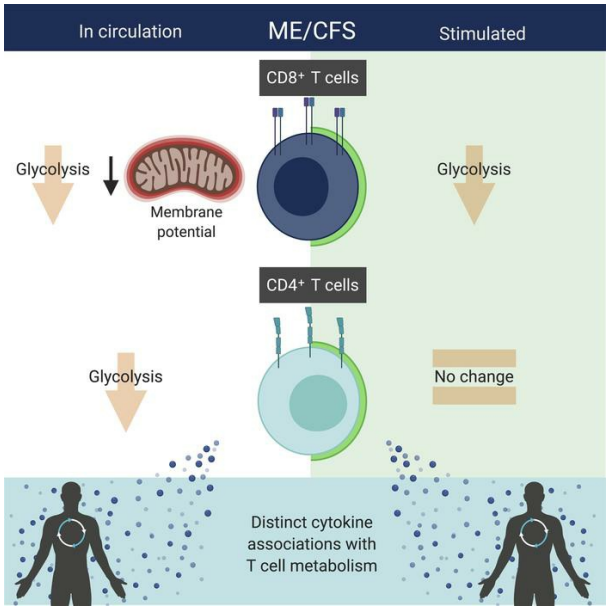
Graphical abstract



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

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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 postexertional malaise. There is little known about the metabolism of specific immune cells in patients with ME/CFS. To investigate immune metabolism in ME/CFS, we isolated CD4$^+$ and CD8$^+$ T cells from 53 patients with ME/CFS 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 had reduced mitochondrial membrane potential compared with those from healthy controls. Both CD4$^+$ and CD8$^+$ T cells from patients with ME/CFS had reduced glycolysis at rest, whereas CD8$^+$ T cells also had reduced glycolysis following activation. Patients with ME/CFS had significant correlations between measures of T cell metabolism and plasma cytokine abundance that differed from correlations seen in 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

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a poorly understood and debilitating disease. An estimated 836,000 to 2.5 million Americans have ME/CFS (1). Patients suffer from severe fatigue, unrefreshing sleep, cognitive impairment, postexertional malaise, pain, gastrointestinal symptoms, and orthostatic intolerance. In addition, many patients with ME/CFS experience flu-like symptoms, such as tender or swollen lymph nodes, fever, muscular pain, headache, and sore throat. Diagnosis is established through the application of 1 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 being 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 this disease (2).

Historically, there have been clustered outbreaks of ME/CFS worldwide, including major outbreaks in the 1980s in the United States in Incline Village, Nevada, and Lyndonville, New York (3). The onset of ME/CFS can either be 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 a cause of ME/CFS.

Characterization of the immune system in patients is an alternative method for exploring 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 patients with ME/CFS, often with conflicting results. However, there have been changes identified in cytokines over the duration of the illness or among patients with differing disease severities (1, 5–7). Cytokines reported to be altered in abundance include TNF-α, TGF-β, IFN-γ, IL-1α, IL-1β, IL-6, and IL-4 (1, 5–10). Some studies have also reported differences in the frequency of CD4$^+$ versus CD8$^+$ T cells, Tregs, and/or memory T cells in patients with ME/CFS (10–16).

Interestingly, NK cells from subjects with ME/CFS have consistently been reported to exhibit reduced cytotoxicity compared with 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.

▶ Related Commentary: p. 1102

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Given the symptoms of fatigue and postexertional malaise, there has also been substantial research into metabolism in patients with ME/CFS. Studies have shown some evidence of oxidative stress and decreased coenzyme Q10 in ME/CFS (19–22). The findings are conflicting with regard to 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 SNPs and ME/CFS, although some SNPs correlated with specific symptoms in patients (26). Others have also found no clinically relevant mitochondrial SNPs in patients with ME/CFS (27, 28).

More recently, multiple metabolomics analyses have been conducted in plasma, serum, and urine from patients with ME/CFS and from healthy controls (29–34). These analyses have shown numerous differentially abundant metabolites in patients with ME/CFS that belong to pathways such as fatty acid metabolism, the citric acid (TCA) cycle, and 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 ROS production can contribute to inflammation and/or autoimmunity (39–42). Conversely, hypometabolism, or 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, in which metabolism and, in turn, effector functions are impaired (46, 47).

Characterization of 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 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. Both $CD4^+$ and $CD8^+$ T cells were isolated from samples collected from 45 healthy controls and 53 patients with ME/CFS. 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 the study population. Study subjects were recruited by Daniel Peterson at Simmaron Research in Incline Village, Nevada, and fulfilled the Canadian Consensus Criteria. A total of 53 patients with ME/CFS and 45 healthy controls were included in the study. The composition of the patient and control groups was similar with regard to sex and age (Table 1). With regard to race, an overwhelming number of the participants were White. Only 1 patient and 1 control subject identified themselves as Hispanic or Latino. Patients had an average illness duration of 21.7 years, and most patients reported being ill for more 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). Of the 53 patients with ME/CFS, 24 reported a gradual onset and 26 reported a sudden onset of the disease; the onset type was unknown for the remaining 3 patients (Table 1). The patients with ME/CFS 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. The patients reported statistically significantly higher scores for all of the specific symptom severity scale items (Table 1). In particular, the patients scored high on impaired memory or concentration, fatigue, muscle tenderness or pain, and postexertional malaise (Table 1). Additionally, the 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. Patients with ME/CFS had statistically significantly lower scores on all dimensions of the SF-36 survey, especially with regard to physical health and vitality (Table 1). Patients reported an average Bell scale score of 37.1 compared with 96.7 for 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 reflected substantial impairment compared with scores for healthy controls and confirmed that our study population had the expected characteristics of the disease.

Both ME/CFS and healthy control subjects were asked a series of questions about gastrointestinal conditions and/or symptoms, comorbidities, and family health history. Thirteen patients with ME/CFS had a previous cancer diagnosis compared with 4 healthy controls ($P = 0.08$) (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI132185DS1). Of 53 patients with ME/CFS, 35 (66%) reported some kind of gastrointestinal symptom, whereas only 8 of 45

Table 1. Study population characteristics and survey responses

		ME/CFS (*n* = 53)	Healthy controls (*n* = 45)	*P* value
Age		50.8 ± 16.2	50.2 ± 17.5	0.86
Illness duration (yr)		21.7 ± 12 (*n* = 50)	–	–
Time from symptoms to diagnosis (yr)		6.7 ± 7 (*n* = 44)	–	–
Bell scale score		37.1 ± 15.7	96.7 ± 6.4	< 0.001
Sex	Male	22	19	1
	Female	31	26	
Race	American Indian and Alaska Native	0	0	
	Asian	0	2	
	Black or African American	0	0	
	White	52	42	
	Two or more races	1	0	
Ethnicity	Hispanic or Latino	1	1	1
	Not Hispanic or Latino	45	39	–
	Unknown	7	5	–
Onset type	Gradual	24	–	–
	Sudden	26	–	–
	Unknown	3	–	–
Triggering event	Known infection	18	–	–
	Viral-like illness	23	–	–
	Other	3	–	–
	Unknown	9	–	–
Specific symptom severity (0 = none, 10 = very severe)	Fatigue	6.9 ± 1.9	1.4 ± 1.5	< 0.001
	Impaired memory or concentration	6.2 ± 2.4	1.3 ± 1.7	< 0.001
	Recurrent sore throat	2.9 ± 2.8	0.3 ± 1.1	< 0.001
	Tender lymph nodes	3.5 ± 3	0.6 ± 1.5	< 0.001
	Muscle tenderness or pain	5.7 ± 2.9	0.9 ± 1.6	< 0.001
	Joint pain	4.8 ± 3.1	1.1 ± 1.8	< 0.001
	Headache	4.3 ± 3.2	0.9 ± 1.1	< 0.001
	Disturbed sleep or waking unrefreshed	7.2 ± 2.4	1.5 ± 1.7	< 0.001
	Postexertional malaise	7.6 ± 2.5	1 ± 1.5	< 0.001
36-Item short-form survey	Physical function	41.8 ± 22.9	88.9 ± 22.5	< 0.001
	Role – physical	6.6 ± 20.3	93.3 ± 18	< 0.001
	Body pain	40.8 ± 24.4	87.8 ± 15	< 0.001
	General health	22.8 ± 14.9	76.7 ± 15.7	< 0.001
	Vitality	18.6 ± 19.3	71.7 ± 16	< 0.001
	Social functioning	31.4 ± 26	90.5 ± 15.3	< 0.001
	Role – emotional	64.8 ± 46	90.7 ± 18.7	0.008
	Mental health	70.1 ± 19.8	83.5 ± 33	0.04
	Physical component score	24.2 ± 7.5	52 ± 8.2	< 0.001
	Mental component score	43.8 ± 11	53.7 ± 10.4	< 0 .001

(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 (IBS) compared with only 6.7% of controls ($P < 0.001$) (Supplemental Table 1). A total of 33 of 53 (62.3%) patients had at least 1 family member with an immune- or inflammation-related disease, whereas only 15 of 45 (33.3%) healthy controls reported the same ($P = 0.008$). This was largely driven by an increased incidence of rheumatoid arthritis and type 1 diabetes for family members of patients with ME/CFS (Supplemental Table 1). No control subjects reported immune or inflammatory disease diagnoses, but 7 patients with ME/CFS reported being diagnosed with at least 1 immune or inflammatory disease ($P = 0.03$) (Supplemental Table 1). Among the patients with ME/CFS, 73.6% indicated having some kind of allergy compared with 48.9% of healthy controls ($P = 0.02$) (Supplemental Table 1).

$CD4^+$ T cell mitochondrial metabolism is not altered in patients with ME/CFS. Blood samples were collected from patients with ME/CFS and healthy control subjects at Simmaron Research (Incline Village, Nevada, USA). Samples from both patients and control subjects were collected over a period of 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, nonmitochondrial respiration, and proton leak. In order to compare resting mitochondrial respiration as well as the capability of patients' 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 cytometric 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 MFI after overnight stimulation (Supplemental Figure 1). We did not always have a sufficient number of T cells to include a subject in every assay. The number of viable PBMCs we received varied among different subjects, and some subjects had a greater frequency of T cells than others, as is expected (53). In particular, the sample size for activated cell assays was reduced in order to perform preferential assays of cells in circulation.

For $CD4^+$ T cells, we detected no significant difference in basal mitochondrial respiration between healthy control and ME/CFS cells at rest (Figure 1A). We observed a small but nonsignificant

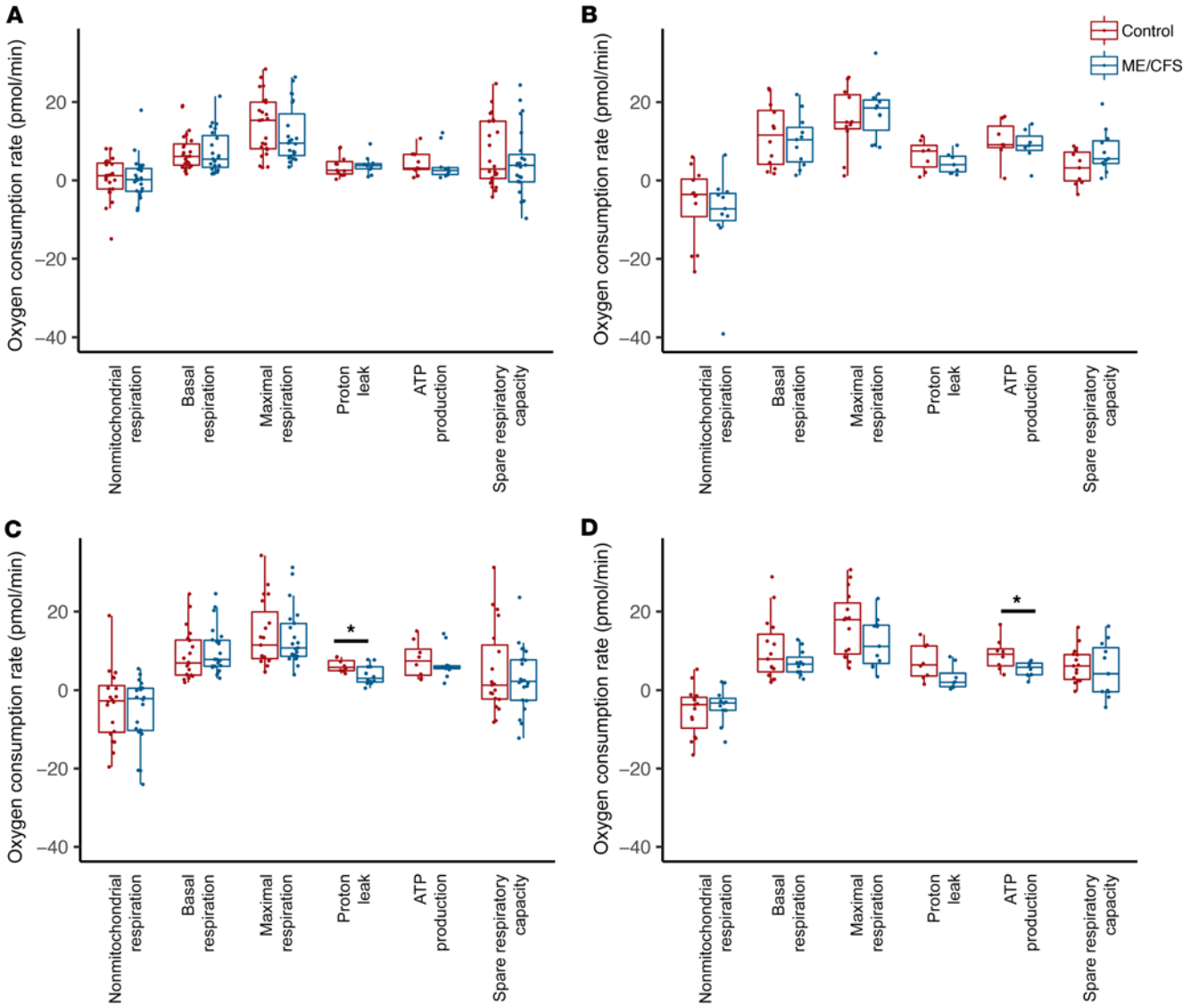

Figure 1. ME/CFS CD8⁺ T cell proton leak and ATP production are reduced compared with healthy control samples. (**A**) Resting mitochondrial respiration parameters for healthy control and ME/CFS CD4⁺ T cells, including nonmitochondrial respiration (n = 24 healthy control samples; n = 23 ME/CFS samples [n = 24/23]), basal respiration (n = 24/23), maximal respiration (n = 24/23), proton leak (n = 11/10), ATP production (n = 11/10), and spare respiratory capacity (n = 24/23). (**B**) Mitochondrial respiration parameters for healthy control and ME/CFS CD4⁺ T cells after overnight stimulation with anti-CD3/anti-CD28 and IL-2, including nonmitochondrial 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). (**C**) Resting mitochondrial respiration parameters for healthy control and ME/CFS CD8⁺ T cells, including nonmitochondrial respiration (n = 20/22), basal respiration (n = 20/22), maximal respiration (n = 19/21), proton leak (n = 8/12), ATP production (n = 8/12), and spare respiratory capacity (n = 19/21). (**D**) Mitochondrial respiration parameters for healthy control and ME/CFS CD8⁺ T cells after stimulation, including nonmitochondrial respiration (n = 15/11), basal respiration (n = 15/11), maximal respiration (n = 15/11), proton leak (n = 8/8), ATP production (n = 8/8), and spare respiratory capacity (n = 15/11). Box plots represent the median (middle line) and 25th and 75th quartiles (bottom and top edges of box). Whiskers represent 1.5 times the IQR and outliers are defined as values beyond whiskers. *P < 0.05 by Wilcoxon rank-sum test.

decrease in maximal respiration in ME/CFS CD4⁺ T cells compared with 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 CD4⁺ T cells at rest (Figure 1A). We detected no significant difference in proton leak between CD4⁺ T cells from patients with ME/CFS versus those from control subjects (Figure 1A). ATP production was slightly reduced in patients' CD4⁺ T cells at rest compared with cells from healthy controls, but there were 2 outliers (Figure 1A).

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

Both healthy control and ME/CFS CD4⁺ T cells displayed higher, although nonsignificant, basal and maximal mitochondri-

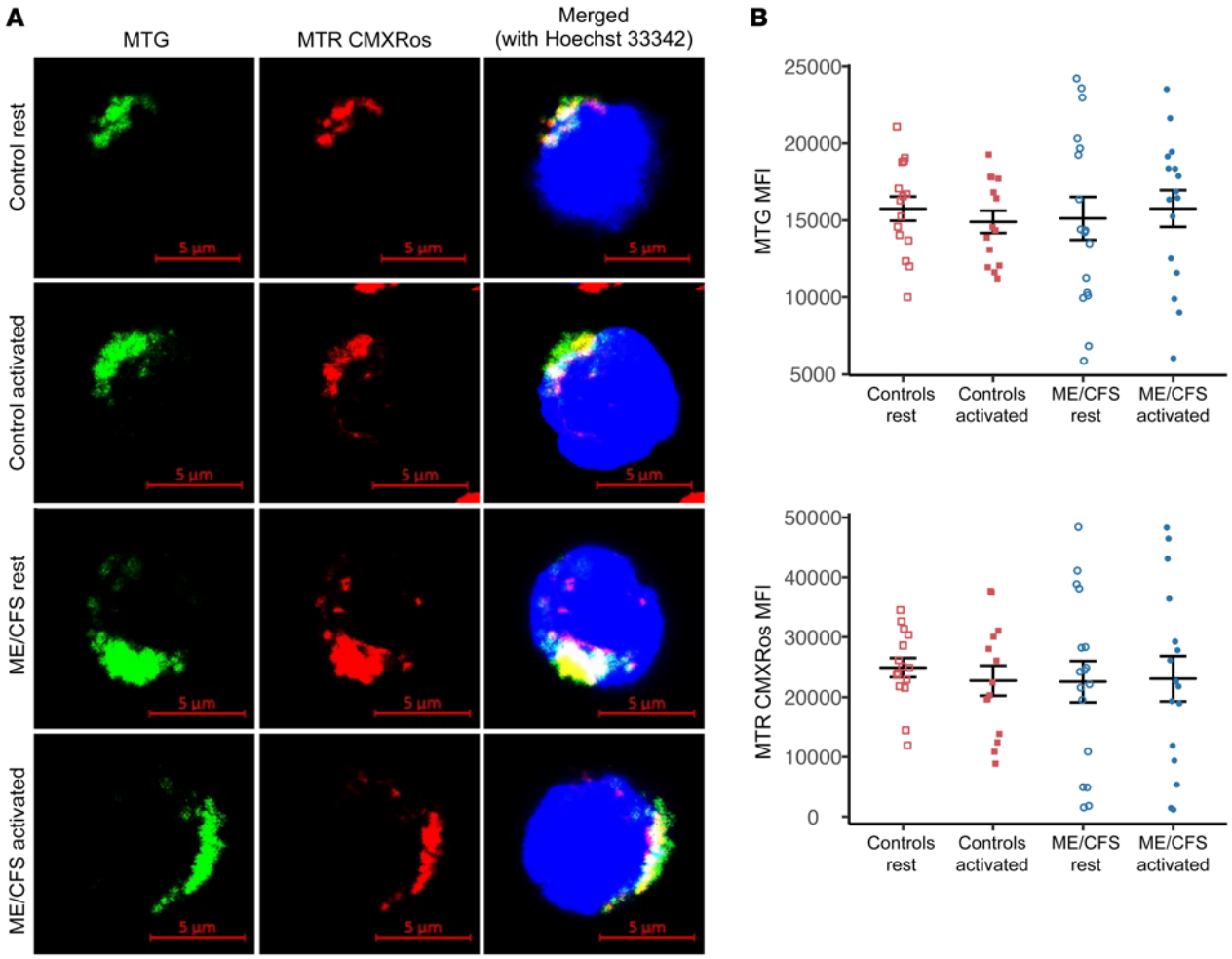

Figure 2. Mitochondrial mass and membrane potential do not differ between healthy control and ME/CFS CD4⁺ T cells. (**A**) MTG, MTR CMXRos, and Hoechst 33342 staining of representative resting and activated control and ME/CFS CD4⁺ T cells. The experiment was conducted 4 times for each condition. Scale bars: 5 µm. (**B**) MTG and MTR CMXRos MFI as determined by flow cytometry in healthy control and ME/CFS CD4⁺ T cells at rest and after overnight activation (n = 15 healthy control samples at rest; n = 14 healthy control samples after activation; n = 17 ME/CFS samples at rest; n = 16 ME/CFS samples after activation). Data represent the mean ± SEM. MTG, MitoTracker Green; MTR, MitoTracker Red.

al respiration following activation compared with cells at rest (Figure 1, A and B). In both groups, we observed a substantial variation in mitochondrial metabolism after activation (Figure 1, A and B). ATP production was markedly increased in healthy control CD4⁺ T cells following activation and trended higher in ME/CFS CD4⁺ T cells (Figure 1, A and B).

Overall, CD4⁺ T cells from patients 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 did 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. We detected no significant difference in basal or maximal mitochondrial metabolism between healthy control and ME/CFS CD8⁺ T cells at rest (Figure 1C). However, proton leak was significantly reduced in CD8⁺ T cells from patients with ME/CFS versus those from healthy controls, suggesting increased mitochondrial efficiency (Figure 1C). ATP production was slightly decreased, but there was high variability in both groups (Figure 1C). We found no difference in spare respiratory capacity between patient and control CD8⁺ T cells at rest (Figure 1C).

After activation, we detected a small reduction in basal mitochondrial respiration in ME/CFS CD8⁺ T cells compared with healthy control cells (Figure 1D). ME/CFS CD8⁺ T cells also showed a small but nonsignificant decrease in maximal respiration (Figure 1D). We observed no difference in spare respiratory capacity between patient and healthy control CD8⁺ T cells after activation (Figure 1D). Proton leak trended lower in stimulated ME/CFS CD8⁺ T cells compared with healthy control cells, which was consistent with the decrease observed 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 1, C and

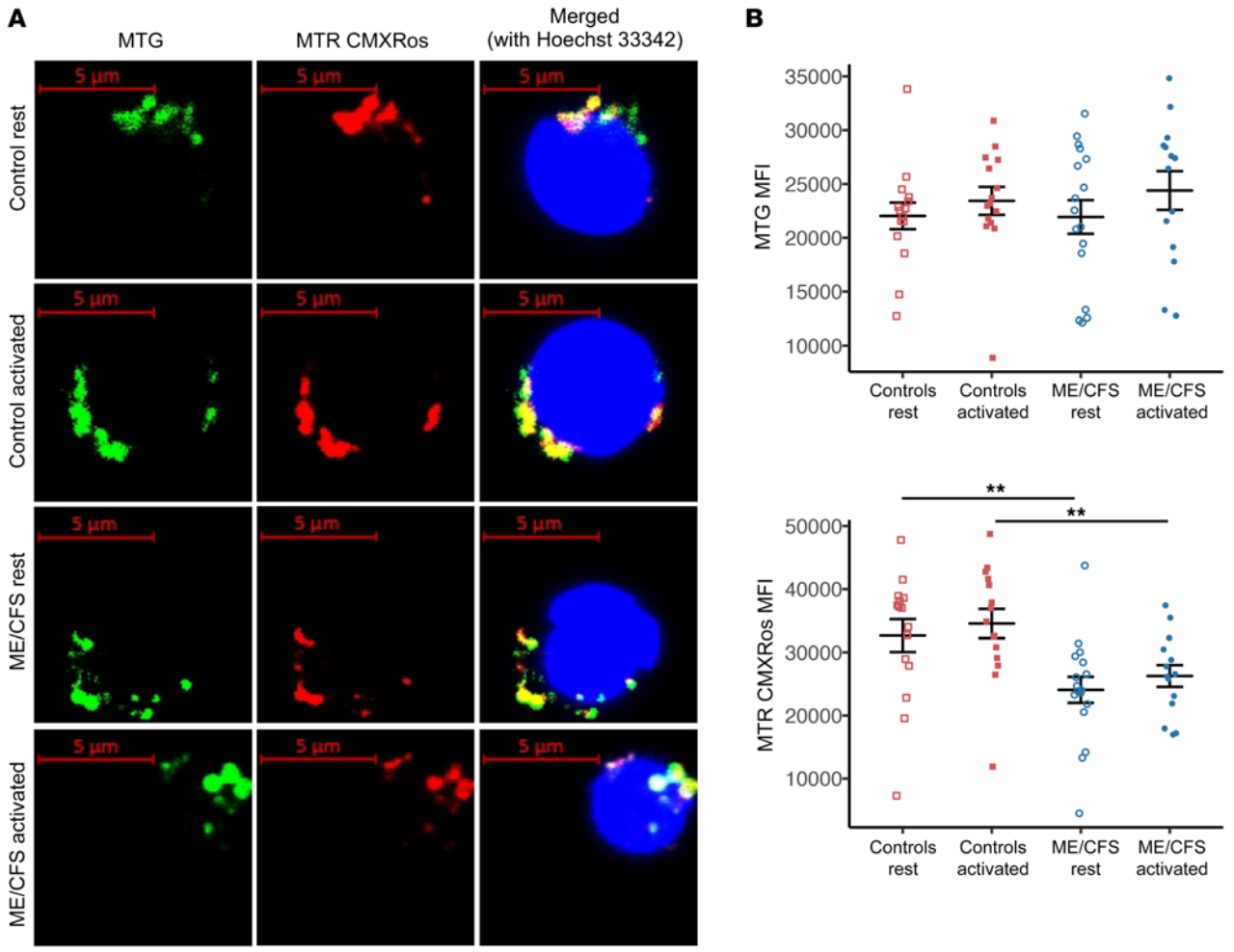

Figure 3. Mitochondrial membrane potential is decreased in CD8⁺ T cells from patients with ME/CFS. (**A**) MTG, MTR CMXRos, and Hoechst 33342 staining of representative resting and stimulated control and ME/CFS CD8⁺ T cells. The experiment was conducted 4 times for each condition. Scale bars: 5 μm. (**B**) MTG and MTR CMXRos MFI as determined by flow cytometry in healthy control and ME/CFS CD8⁺ T cells at rest and after overnight activation (n = 15 healthy control samples at rest; n = 15 healthy control samples after activation; n = 17 ME/CFS samples at rest; n = 14 ME/CFS samples after activation). Data represent the mean ± SEM. $^{**}P < 0.01$ by Kruskal-Wallis followed by Dunn's test with FDR-based multiple testing correction. MTG, MitoTracker Green; MTR, MitoTracker Red.

D). Meanwhile, ME/CFS CD8⁺ T cells showed either no change or a decrease in basal mitochondrial metabolism after activation compared with resting cells (Figure 1, C and D). The same pattern was found in both maximal respiration and ATP production (Figure 1, C and D). Thus, ME/CFS CD8⁺ T cells may be less able to induce metabolism following activation.

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

CD4⁺ T cell mitochondrial mass and membrane potential do 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. We stained both CD4⁺ and CD8⁺ T cells with MitoTracker Green (MTG) and MitoTracker Red (MTR) CMXRos simultaneously. MTG indicates mitochondrial mass, whereas MTR CMXRos is sensitive to mitochondrial membrane potential. We evaluated mitochondria by confocal microscopy as well as flow cytometry. For confocal microscopy, cells were also stained with Hoechst 33342 to visualize nuclei. Both the microscopic and flow cytometric experiments were conducted after overnight resting or stimulation of cells to determine how T cell mitochondria from patients and controls respond to activation.

Confocal microscopy revealed no difference in MTG or MTR CMXRos staining of CD4⁺ T cells (Figure 2A). Both at rest and following activation, the MitoTracker dyes costained well in merged images of CD4⁺ T cells from patients and controls (Figure 2A). We observed no morphological differences in mitochondria within CD4⁺ T cells from patients and controls, although the fluorescence image resolution was limited (Figure 2A).

Flow cytometry revealed no significant difference in MTG MFI between healthy control and ME/CFS CD4⁺ T cells either at rest or after stimulation, indicating that there was no difference in mitochondrial mass in either state (Figure 2B). Furthermore,

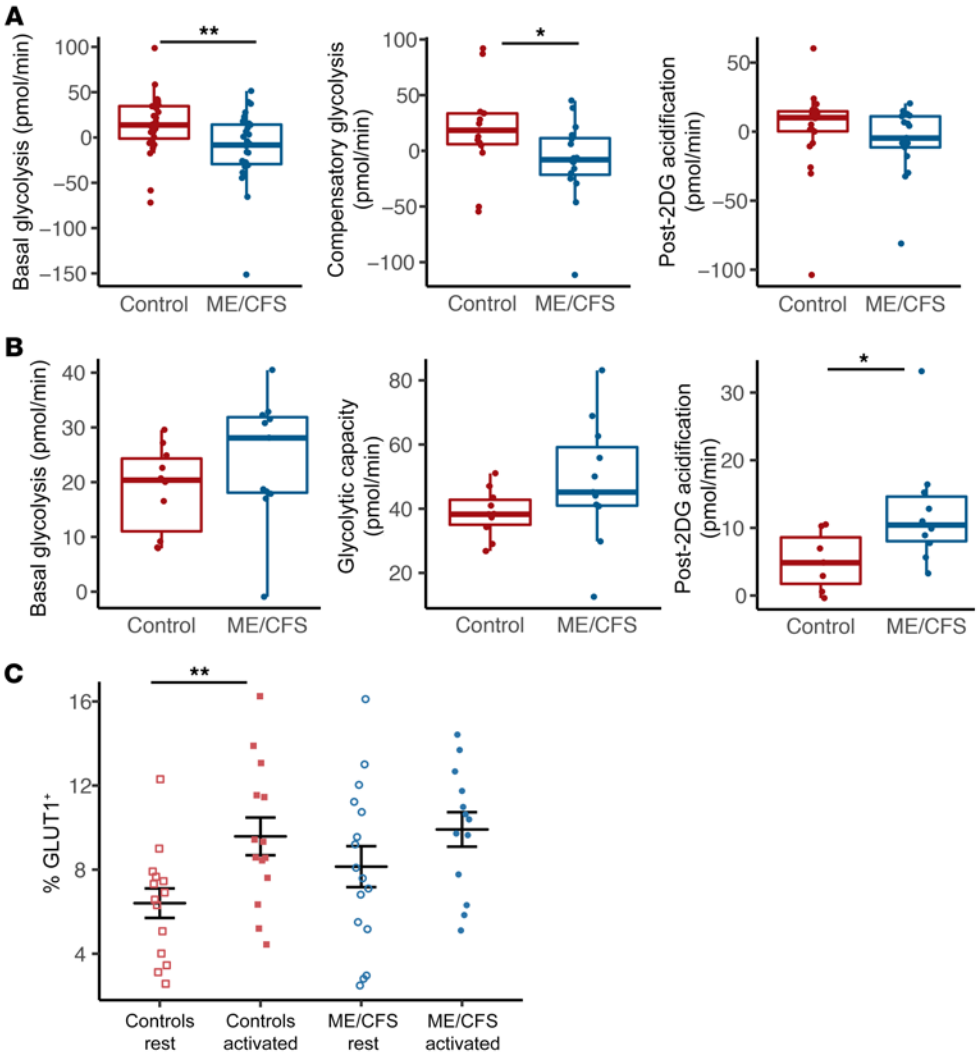

Figure 4. Basal glycolysis is reduced in ME/CFS CD4⁺ T cells. (**A**) Resting glycolysis measurements from Seahorse extracellular flux analysis of healthy control and ME/CFS CD4⁺ T cells, including basal glycolysis (n = 28), compensatory glycolysis (n = 15 healthy control samples; n = 16 ME/CFS samples [n = 15/16]), and post-2DG acidification (n = 22/17). (**B**) Glycolysis measurements in stimulated healthy control and ME/CFS CD4⁺ T cells, including basal glycolysis (n = 10/11), glycolytic capacity (n = 10/11), and post-2DG acidification (n = 7/10). (**C**) Percentage of GLUT1⁺ cells in resting and activated CD4⁺ T cells from patients with ME/CFS and healthy controls (n = 14 healthy control samples at rest; n = 14 healthy control samples after activation; n = 16 ME/CFS samples at rest; n = 13 ME/CFS samples after activation). Box plots represent the median (middle line) and 25th and 75th quartiles (bottom and top edges of box). Whiskers represent1.5 times the IQR) and outliers are defined as values beyond the whiskers. For dot plots, data represent the mean ± SEM. *P < 0.05; **P < 0.01, by Wilcoxon rank-sum test (**A** and **B**) and Kruskal-Wallis followed by Dunn's test with FDR-based multiple testing correction (**C**).

we observed no significant differences in MTR CMXRos MFI between healthy control and ME/CFS CD4⁺ T cells at rest or after activation (Figure 2B). As such, there was also no apparent difference in mitochondrial membrane potential in CD4⁺ T cells from patients compared with those from controls.

ME/CFS CD8⁺ T cells have decreased mitochondrial membrane potential. We also visualized CD8⁺ T cells via confocal microscopy to assess mitochondrial morphology, mass, and membrane potential. MTG staining revealed no difference in mitochondria in cells from healthy controls versus those from patients with ME/CFS (Figure 3A). However, at 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 staining (Figure 3A). Meanwhile, CD8⁺ T cells from healthy controls showed an even costaining of MTG and MTR CMXRos, similar to that observed with CD4⁺ T cells (Figure 3A). We found no observable difference in mitochondrial morphology between healthy control and ME/CFS CD8⁺ T cells in either state. Further imaging experiments, particularly by electron microscopy, will be necessary to determine whether there are differences not detectable by fluorescence microscopy.

We then used flow cytometry to quantify fluorescence of the 2 mitochondrial dyes in CD8⁺ T cells. Consistent with our microscopic images, we observed no significant difference in MTG MFI between healthy control and ME/CFS CD8⁺ T cells at rest (Figure 3B). We noted a small but nonsignificant increase in MTG MFI following overnight stimulation of cells from both control subjects and patients (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 with healthy control CD8⁺ T cells (Figure 3B). Thus, CD8⁺ T cells from patients had no difference in mitochondrial mass in either state but had decreased mitochondrial membrane potential compared with healthy control cells.

ME/CFS CD4⁺ T cell glycolysis is reduced at rest compared with 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 glycolysis in healthy control cells. Additionally, we sought to determine 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 using the Seahorse XFe96 extracellular flux analyzer. For resting

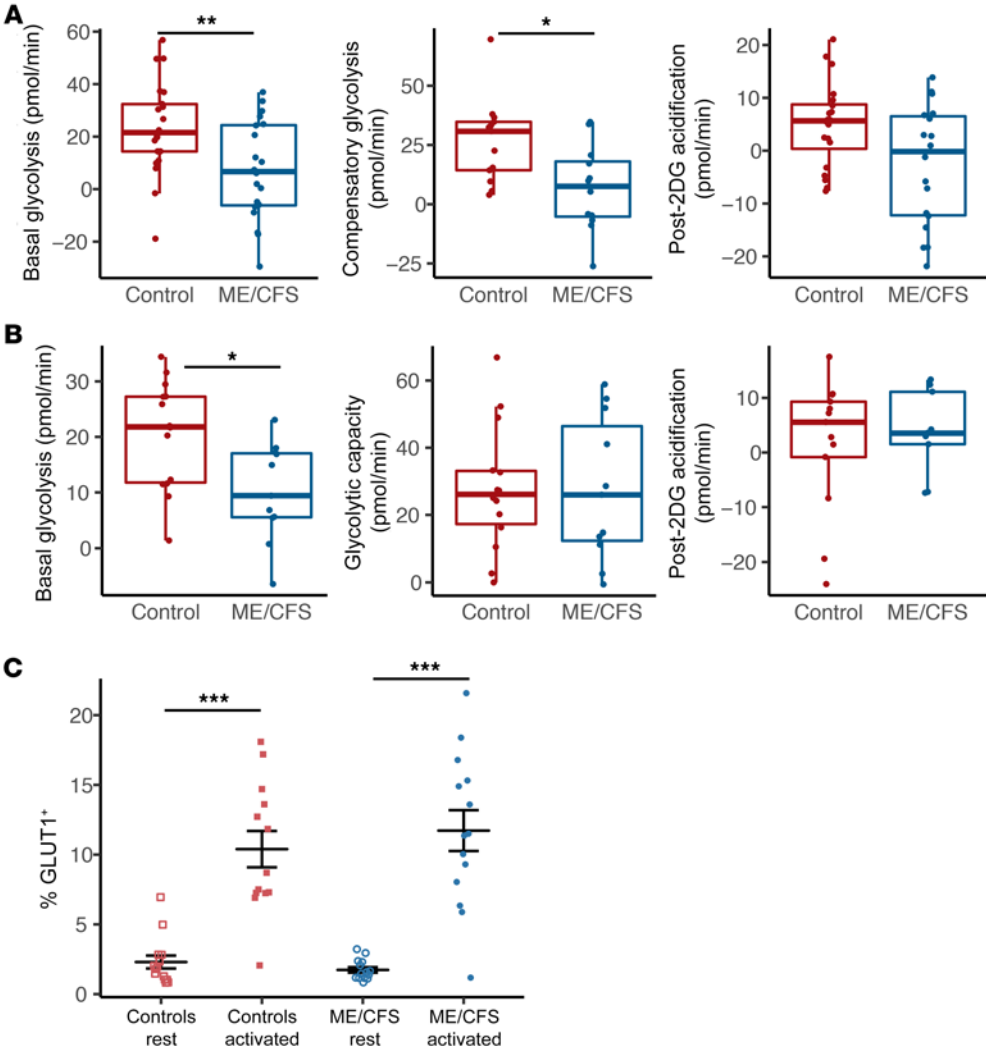

Figure 5. Basal glycolysis is reduced in ME/CFS CD8⁺ T cells. (**A**) Resting glycolysis measurements from Seahorse extracellular flux analysis of healthy control and ME/CFS CD8⁺ T cells, including basal glycolysis (n = 21 healthy control samples; n = 20 ME/CFS samples [n = 21/20]), compensatory glycolysis (n = 13/12), and post-2DG acidification (n = 20/18). (**B**) Glycolysis measurements in stimulated healthy control and ME/CFS CD8⁺ T cells, including basal glycolysis (n = 14/11), glycolytic capacity (n = 14/11), and post-2DG acidification (n = 13/9). (**C**) Percentage of GLUT1⁺ cells in resting and activated CD8⁺ T cells from patients with ME/CFS and healthy controls (n = 14 healthy control samples at rest; n = 13 healthy control samples after activation; n = 14 ME/CFS samples at rest; n = 14 ME/CFS samples after activation). Box plots represent the median (middle line) and 25th and 75th quartiles (bottom and top edges of box). Whiskers represent 1.5 times the IQR and outliers are defined as values beyond the whiskers. For dot plots, data represent the 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 FDR-based multiple testing correction (**C**).

T cells, we analyzed glycolysis with a Seahorse Glycolytic Rate Assay, which provides quantitative measurements of basal glycolysis, compensatory glycolysis, and post–2-deoxyglucose (post-2DG) acidification. Given the limited number of cells we had available for assaying glycolysis after activation, we instead used 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 2DG and 5 measurements. Because we used 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, we found that basal glycolysis was significantly lower in cells from patients with ME/CFS compared with those from healthy controls (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 with healthy control CD4⁺ T cells (Figure 4A). Interestingly, we found no difference in basal glycolysis rates between healthy control and patient CD4⁺ T cells after overnight stimulation, suggesting that activation was sufficient to overcome the decrease in 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 the small increase in glycolytic capacity observed in patients' cells (Figure 4B). Post-2DG acidification was significantly higher in ME/CFS CD4⁺ T cells compared with that in CD4⁺ T cells from healthy controls, despite the slight decrease in post-2DG acidification observed in resting cells (Figure 4B). This may indicate increased extracellular acidification from nonglycolytic sources in activated ME/CFS CD4⁺ T cells. Additionally, this finding may explain the small increase in glycolytic rates compared with control cells.

Between rest and stimulation, we found that basal glycolysis increased in both healthy control and ME/CFS CD4⁺ T cells, but this difference was only substantial in ME/CFS cells (Figure 4, A and B). Post-2DG acidification did not differ in healthy control CD4⁺ T cells from rest to activation but was higher in ME/CFS CD4⁺ T cells after activation, indicating increased extracellular sources of acidification following stimulation (Figure 4, A and B).

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

ME/CFS CD8$^+$ T cell glycolysis is decreased at rest and after activation. We next investigated glycolysis in CD8$^+$ T cells. As with CD4$^+$ T cells, we found that 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 after activation compared with healthy control CD8$^+$ T cells (Figure 5B). We observed 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 cells from both groups (Figure 5B).

There were minimal changes in glycolysis between rest and activation for both healthy control and ME/CFS CD8$^+$ T cells (Figure 5, A and B). Interestingly, we detected a slight, nonsignificant 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 5, A and 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 with mitochondrial respiration.

We also quantified GLUT1$^+$ cells via flow cytometric analysis 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, we again observed no significant differences in the abundance of GLUT1$^+$ cells between healthy control and ME/CFS cells, either at rest or after activation (Figure 5C). Nevertheless, we detected a lower percentage of GLUT1$^+$ cells in patient CD8$^+$ T cells at rest than in healthy control CD8$^+$ T cells (Figure 5C).

Plasma cytokines uniquely correlate with T cell metabolism in patients with ME/CFS. In addition to PBMCs, we collected EDTA plasma samples 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. Of the 48 cytokines and chemokines measured, 44 were detected. We compared cytokine abundance in plasma of patients with ME/CFS and healthy controls and found no significant differences between the groups for any of the 44 cytokines detected (Supplemental Table 2).

Although we did not detect differences in plasma cytokine abundance between patients and controls, we were interested in determining whether T cell metabolism correlated with the levels of plasma cytokines or survey data. To do this, we performed a Spearman's correlation test with FDR correction for multiple testing. Correlations with a FDR-adjusted P value (q value) below 0.01 were considered significant. We also analyzed correlations in patients and controls separately to determine 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 found no significant correlations between T cell metabolism and survey data in patients with ME/CFS or healthy controls. Additionally, T cell metabolism was not significantly different in patients with IBS or IBD compared with those without either disease. However, we found a number of significant correlations between plasma cytokines and metabolism. Most interestingly, these correlations were unique in patients with ME/CFS compared with the control group. In patients with ME/CFS, 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 SCGF-β (Figure 6, A–E). None of these correlations were significant in healthy control subjects (Figure 6, A–E). Conversely, we found a significant positive correlation between resting basal glycolysis in CD8$^+$ T cells and abundance of 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 macrophage-CSF (M-CSF) and TNF-α (Figure 6, G and H). Interestingly, we observed the opposite trends in healthy control cells (Figure 6, G and H). Following stimulation, patient CD8$^+$ T cell post-2DG acidification and glycolytic capacity both correlated negatively with M-CSF abundance (Figure 6, I and J). In healthy controls, the same relationships existed but were not significant (Figure 6, I and J). As many of these cytokines are proinflammatory, it is surprising that their abundance correlated with reduced metabolism in ME/CFS CD8$^+$ T cells.

We detected 4 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 the proinflammatory cytokine IL-17 (Figure 7, A and B). Meanwhile, in patients with ME/CFS, these correlations were nonsignificant, and maximal respiration was negatively associated with IL-17 (Figure 7, A and B). We observed a significant negative correlation between plasma IL-9 and activated CD4$^+$ T cell basal respiration that was not present in patients with ME/CFS (Figure 7C). Finally, we detected a significant negative correlation between resting CD8$^+$ T cell spare mitochondrial respiratory capacity and G-CSF in healthy controls, but not in patients with ME/CFS (Figure 7D). Overall, patients with ME/CFS and healthy controls have unique correlations between plasma cytokines and T cell metabolism that 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).

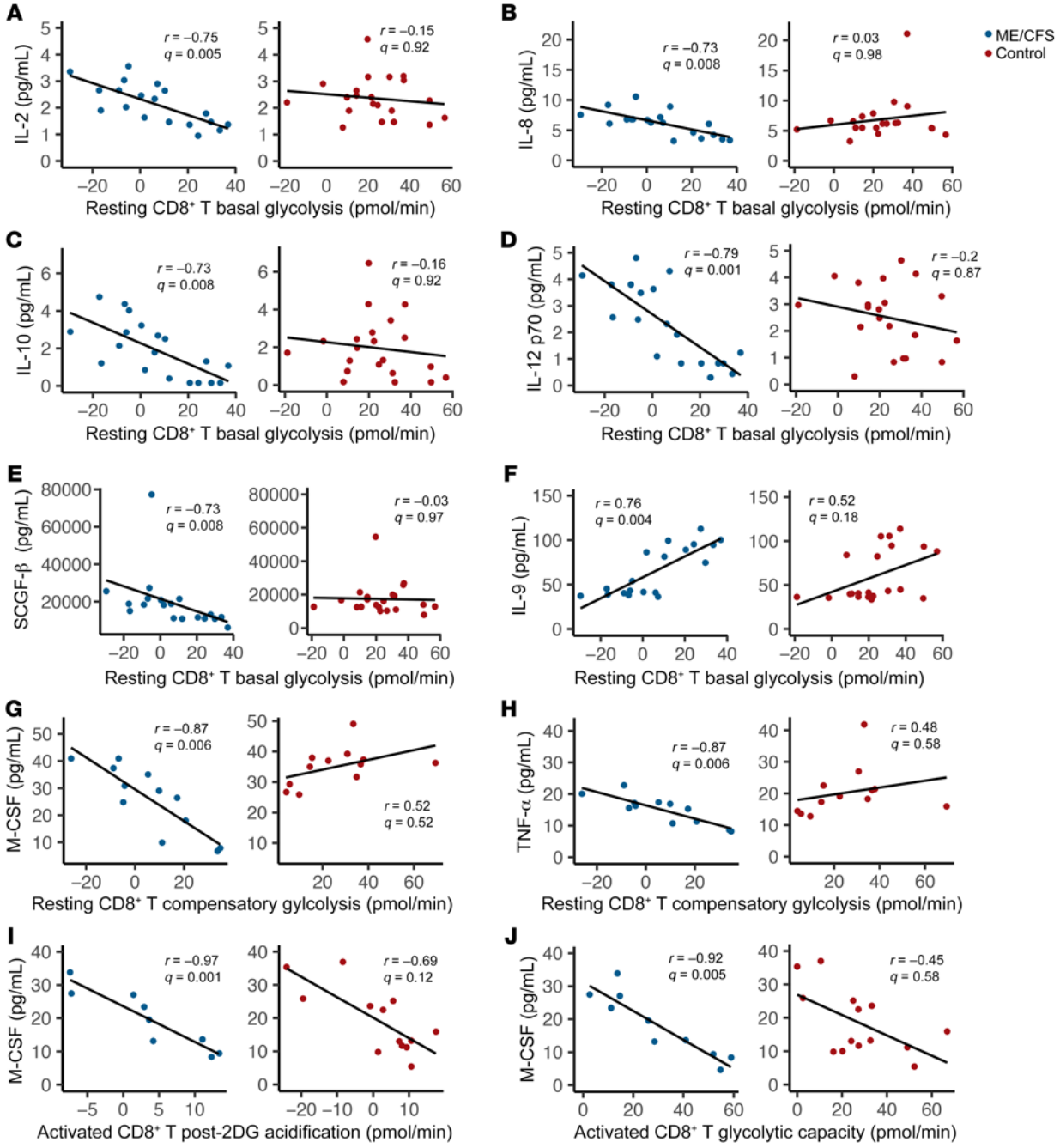

Figure 6. Plasma cytokines are uniquely correlated with T cell metabolism in patients with ME/CFS. Significant correlations between plasma cytokines and cellular metabolism in patients and nonsignificant correlations in healthy controls. Correlations between resting CD8+ T cell (CD8+ T) basal glycolysis and (**A**) IL-2 (n = 19 patients; n = 21 controls), (**B**) IL-8 (n = 18 patients; n = 19 controls), (**C**) IL-10 (n = 19 patients; n = 21 controls), (**D**) IL-12 p70 (n = 19 patients; n = 21 controls), (**E**) SCGF-β (n = 19 patients; n = 20 controls), (**F**) and IL-9 (n = 19 patients; n = 21 controls); between resting CD8+ T cell compensatory glycolysis and (**G**) M-CSF (n = 12 patients; n = 12 controls) and (**H**) TNF-α (n = 12 patients; n = 12 controls); (**I**) between activated CD8+ T cell post-2DG acidification and M-CSF (n = 9 patients; n = 13 controls); and (**J**) between activated CD8+ T cell glycolytic capacity and M-CSF (n = 10 patients; n = 14 controls). All correlations were evaluated using a Spearman's correlation test with FDR-based multiple testing correction, where a q value of less than 0.01 was considered significant.

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

Our study population was well balanced with regard to sex, which allowed us to characterize T cells in both male and female

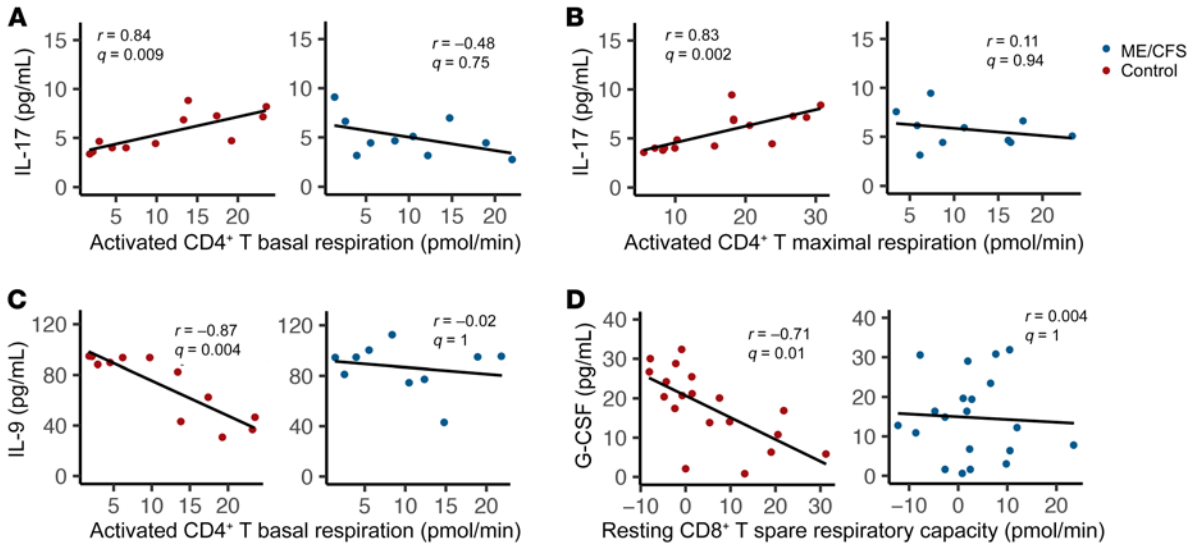

Figure 7. Plasma cytokines correlate with T cell metabolism in healthy controls. Significant correlations between plasma cytokines and cellular metabolism in healthy control subjects and nonsignificant correlations in patients. Correlations between (**A**) activated CD4⁺ T cell basal respiration and IL-17 in control subjects (n = 12) and patients (n = 10); (**B**) between activated CD4⁺ T cell maximal respiration and IL-17 in control subjects (n = 15) and patients (n = 10); (**C**) between activated CD4⁺ T cell basal respiration and IL-9 in control subjects (n = 12) and patients (n = 10); and (**D**) between resting CD8⁺ T cell spare respiratory capacity and G-CSF in control subjects (n = 19) and patients (n = 20). All correlations were evaluated using a Spearman's correlation test with FDR-based multiple testing correction, where a q value of less than 0.01 was considered significant.

subjects. Almost all subjects were White, which is common in ME/CFS studies but not necessarily reflective of the true patient population (1). Our population was composed mainly of patients with long-duration disease, which is not surprising, given that the average time to diagnosis reported was nearly 7 years. Thus, an analysis of T cell metabolism in patients with short- versus long-duration disease was not possible in this study, but a future investigation including more patients with short-duration disease may yield additional insights.

Given the evidence of a dysfunctional immune system in patients with ME/CFS and the increasing appreciation of the role that 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 with healthy control PBMCs, but our study is the first to our knowledge to study specific T cell metabolism in ME/CFS (51). We characterized the 2 main energy-producing pathways — oxidative phosphorylation and glycolysis — in cells and their underlying cellular components.

Our study of mitochondrial respiration revealed that ME/CFS CD4⁺ T cells had no dysfunction in mitochondrial metabolism or any difficulty in increasing respiration in response to activation. The wide variation in many measures of metabolism was expected, but limited our ability to differentiate 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 had increased mitochondrial respiration after activation (37, 56). There were also no differences in mitochondrial mass or membrane potential between ME/CFS and healthy control CD4⁺ T cells. Surprisingly, we observed 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 only observed a significant reduction in proton leak in ME/CFS cells at rest compared with healthy control cells. A decrease in proton leak would suggest an increase in mitochondrial respiratory efficiency, yet basal respiration, maximal respiration, and ATP production were not significantly different in ME/CFS CD8⁺ T cells compared with healthy control cells. After activation, we observed a similar phenotype. As a whole, basal respiration in ME/CFS CD8⁺ T cells did not increase to the same extent as that in healthy control cells following stimulation, suggesting an impairment in the ability of these ME/CFS CD8⁺ T cells to undergo metabolic reprogramming.

Our study of mitochondria in CD8⁺ T cells through imaging revealed a significant reduction in mitochondrial membrane potential in ME/CFS CD8⁺ T cells both at rest and after activation. This was surprising in light of the observed decrease in proton leak in patients' 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 at rest than did CD8⁺ T cells from healthy control subjects. Both healthy control and ME/CFS CD8⁺ T cells had expected increases in mitochondrial mass and membrane potential after activation, although this difference was nonsignificant and insufficient 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 the impaired metabolic response to activation and the greater observable differences in mitochondrial respiration in stimulated cells. A decrease in 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 patients with ME/CFS, 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, we observed no significant difference in CD4+ T cell basal glycolysis or glycolytic capacity, indicating that activation was sufficient to overcome resting defects in glycolysis. ME/CFS and healthy control CD4+ T cells both had 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 with healthy controls after activation. Along with our findings in CD8+ T cell mitochondrial respiration after activation, this significant decrease in glycolysis supports an impairment in the ME/CFS CD8+ T cell metabolic response to activation. Nevertheless, there were minimal changes in the rates of glycolysis in patient and healthy control CD8+ T cells 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), with no significant differences between cells from patients and control subjects. We detected a slight increase in the percentage of ME/CFS CD4+ T cells that are GLUT1+ compared with controls. However, we observed a small reduction in the percentage of ME/CFS CD8+ T cells that are GLUT1+ at rest compared with control cells, which is consistent with the decrease in glycolysis at rest. A previous study of T cell exhaustion demonstrated increased rather than decreased GLUT1 expression with impaired glycolysis (61). Additionally, glucose may be used for alternative pathways that 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 patients with ME/CFS compared with controls. ME/CFS CD8+ T cell glycolysis had multiple significant correlations with cytokines that are known to be proinflammatory or with growth factors (63–65). This is especially interesting, given the reduced basal glycolysis we observed in patients with ME/CFS. 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 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 patients with ME/CFS.

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 patients with breast cancer, 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–induced improvement of Treg 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 finding suggests that the relationship between proinflammatory cytokines in plasma and T cell metabolism is altered in patients with ME/CFS and implicates specific cytokines in ME/CFS that merit further investigation.

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 had altered mitochondrial membrane potential and an impaired metabolic response to activation. Both CD4+ and CD8+ T cells had significant reductions in glycolysis. This hypometabolism in T cells aligns with other findings of hypometabolism in ME/CFS cells (50, 51, 59). Furthermore, patients with ME/CFS appeared to have altered relationships between plasma cytokine abundance and T cell metabolism, in which proinflammatory cytokines unexpectedly correlated 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 infectious agent has not yet been identified. A high priority moving forward will be to determine 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 (Incline Village, Nevada, USA). The subjects with ME/CFS were established patients of Daniel Peterson. Healthy controls and patients with ME/CFS completed approved questionnaires including an SF-36, a Bell scale, a specific symptom inventory, and additional questions regarding symptoms, comorbidities, and family health history.

Sample collection and processing. Blood (80 mL) from each subject was collected into EDTA tubes and immediately processed for collection of aliquots of whole blood, plasma, and PBMCs. Briefly, EDTA tubes were spun at 500 ×*g* for 5 minutes, and plasma was removed and stored at –80°C. Blood was diluted 1:2 in PBS and layered over Histopaque 1077 (Sigma-Aldrich) in 50-mL SepMate tubes (STEMCELL Technologies). SepMate tubes were spun at 1200 ×*g* 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 ×*g* to remove platelets and then a second time at 300 ×*g* 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 1 month of isolation to Cornell University. PBMCs were stored long term in liquid nitrogen, and plasma was stored at –80°C.

Immune cell isolation. Specific immune cell subsets were isolated using STEMCELL EasySep kits on a STEMCELL 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 and then strained through a 37-μm cell strainer to remove any remaining clumps. After a second wash, cells were isolated using the EasySep Human CD19 Positive Selection II Kit, the EasySep Human CD56 Positive Selection II Kit, the EasySep Human CD8 Positive Selection II Kit, and the EasySep Human $CD4^+$ T Cell Isolation Kit, sequentially, and following the manufacturer's instructions. Isolated cells were frozen and stored as described above.

Extracellular flux analysis. Extracellular flux experiments were conducted on a Seahorse XFe96 (Agilent Technologies) at Dartmouth-Hitchcock Medical Center (Lebanon, New Hampshire, USA) or at the Cornell University Biotechnology Resource Center. Cells analyzed at Dartmouth-Hitchcock Medical Center were transported on dry ice and 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, 10 mM glucose, 1 mM 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) (Corning, Thermo Fisher Scientific). The plate was spun at 300 ×*g* for 1 minute with no brake to adhere cells. Assays were run using a Seahorse Mito Stress Kit, a Seahorse Glycolytic Rate Assay, or a Mito Stress Kit, with an added final injection of 2DG with 5 measurements. Drugs were injected at the following final concentrations: 1 μM oligomycin, 1 μM FCCP, 0.5 μM rotenone/antimycin A, and 50 mM 2DG. For activation assays, cells were cultured in complete RPMI overnight prior to the assay, with addition of STEMCELL Technologies' stimulating reagents ImmunoCult (25 μL/mL) (anti-CD3 and anti-CD28) and IL-2 (80 U/mL).

Seahorse data were first normalized on the basis of cell-seeding density per 100,000 viable cells using Wave Software. Data were 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, the glycolysis-specific proton efflux rate was calculated as described by Agilent Technologies (70). Data were only retained if at least 2 replicates gave quality data for a given variable. Basal respiration and maximal respiration were required to be positive (above nonmitochondrial respiration levels), whereas 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 cytometric analysis was performed on a BD FACS Aria II in 5-mL round-bottom polystyrene tubes or on a 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 cytometric analysis was conducted immediately following the staining protocol. Data analysis was conducted using Kaluza Software (Beckman Coulter). $CD8^+$ T cells were first gated as viable, and then $CD3^+CD8^+$ cells were gated for further analysis. The percentage of $GLUT1^+$ cells and the MFI of MTG, MTR CMXRos, and CD69 were calculated in this cell population. For $CD4^+$ T cells, $CD4^+$ or $CD3^+CD4^+$ cells were gated following live/dead exclusion. Then, the percentage of $GLUT1^+$ cells and the MFI of MTG, MTR CMXRos, and CD69 were determined.

Confocal microscopy. Confocal microscopic imaging of immune cells was completed at the Cornell Biotechnology Resource center using 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 (2 μg/mL), MTG (100 nM), and MTR CMXRos (50 nM). Cells were then washed in RPMI 1640 media without FBS and moved onto a glass-bottom 24-well MatTek plate coated with 22.4 mg/mL Cell-Tak. The plate was spun down at 300 ×*g* for 1 minute with no brake. Images were taken immediately following staining using a ×63 oil immersion objective with 12-bit depth, bidirectional scanning, and a ×3.6 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. A total of 48 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). 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-1Rα; 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; IFN family: IFN-α2, IFN-γ; TNF: TNF-α, TNF-β, TNF-related, apoptosis-inducing ligand (TRAIL); GM-CSF; CC chemokines: monocyte chemoattractant protein 1 (MCP-1, also known as CCL2), macrophage inflammatory protein 1-α/β (MIP-1α, also known as CCL3), MIP-1β (also known as CCL4), regulated upon activation, normal T cell expressed, and secreted RANTES (CCL5), monocyte chemotactic protein 3 (MCP-3, also known as CCL7), eotaxin (CCL11), cutaneous T cell–attracting chemokine (CTACK, also known as CCL27); CXC chemokines: growth-regulated protein α GROα (CXCL1), IL-8 (CXCL8), monokine induced by IFN-γ (MIG, also known as CXCL9), IP-10 (CXCL10), stromal cell–derived factor 1-α (SDF-1, also known as CXCL12); PDGF family/VEGF subfamily: PDGFBB, VEGFA; and macrophage migration inhibitory factor (MIF), lymphocyte chemoattractant factor IL-16 (LCF), stem cell growth factor β (SCGF-β), FGF basic (FGFb), β nerve growth factor (βNGF), HGF, stem cell factor (SCF), macrophage-CSF (M-CSF), and granulocyte-CSF (G-CSF). Concentrations were calculated using xPONENT 4.2 software for MAGPIX (Luminex). A 5-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 midpoint 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 using a Wilcoxon rank-sum test, whereas dichotomous response questions

were analyzed using a χ^2 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 comparisons, and *P* values were adjusted using FDR-based multiple testing correction. For comparisons of 4 groups (ME/CFS at rest, control cells at rest, ME/CFS activated cells, control activated cells), statistical testing was conducted using a Kruskal-Wallis test followed by Dunn's test with FDR-based multiple testing correction. For correlation testing, Spearman's correlation tests were performed on all data, followed by a FDR-based multiple testing correction. A *q* value of less than 0.01 was considered significant.

Study approval. Human subject experimental protocols were approved by the Western Institutional Review Board, Olympia, Washington, USA (protocol 20161887). All subjects gave written informed consent before their participation in this study.

Author contributions

AHM, MRH, JM, and LG designed the study. AHM, JM, and LG conducted the experiments. AHM, JM, LG, and MRH analyzed the data. AHM, MRH, JM, and LG wrote the manuscript. DLP diagnosed the study subjects. MM and CGG were responsible for clinical coordination.

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