Perturbation of effector and regulatory T cell subsets in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS)

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Abstract

18
19 Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a debilitating disorder of
20 unknown etiology, and diagnosis of the disease is largely based on clinical symptoms. We
21 hypothesized that immunological disruption is the major driver of this disease and analyzed a
22 large cohort of ME/CFS patient or control blood samples for differences in T cell subset
23 frequencies and functions. We found that the ratio of CD4+ to CD8+ T cells and the proportion
24 of CD8+ effector memory T cells were increased, whereas NK cells were reduced in ME/CFS
25 patients younger than 50 years old compared to a healthy control group. Remarkably, major
26 differences were observed in Th1, Th2, Th17 and mucosal-associated invariant T (MAIT) T cell
27 subset functions across all ages of patients compared to healthy subjects. While CCR6+ Th17
28 cells in ME/CFS secreted less IL-17 compared to controls, their overall frequency was higher.
29 Similarly, MAIT cells from patients secreted lower IFNγ, GranzymeA and IL-17 upon
30 activation. Together, these findings suggest chronic stimulation of these T cell populations in
31 ME/CFS patients. In contrast, the frequency of regulatory T cells (Tregs), which control
32 excessive immune activation, was higher in ME/CFS patients. Finally, using a machine learning
33 algorithm called random forest, we determined that the set of T cell parameters analyzed could
34 identify more than 90% of the subjects in the ME/CFS cohort as patients (93% true positive rate
35 or sensitivity). In conclusion, these multiple and major perturbations or dysfunctions in T cell
36 subsets in ME/CFS patients suggest potential chronic infections or microbiome dysbiosis. These
37 findings also have implications for development of ME/CFS specific immune biomarkers and
38 reveal potential targets for novel therapeutic interventions.

Introduction

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a highly debilitating illness often characterized by symptoms such as post-exertional malaise or severe fatigue not alleviated by rest, muscle and joint pain, sleep problems, hypersensitivity to sensory stimuli, and gastrointestinal symptoms (Holgate et al., 2011;Yancey and Thomas, 2012;Bested and Marshall, 2015). ME/CFS is thought to afflict up to two million individuals in the US alone, with severe long-term disability and negative impacts on quality of life (Valdez et al., 2018). The specific cause and biological basis of ME/CFS remain elusive. Lack of understanding of biological pathways leading to this syndrome is also a major impediment in developing specific therapies and reliable biomarker-based diagnostic tests (Klimas et al., 2012).

While the causes of ME/CFS are likely to be multifactorial, a common history of initial infectious agents, including viral (e.g. EBV) and bacterial (e.g. Lyme Disease) infections, have been associated with triggering the disease (Hickie et al., 2006;Katz et al., 2009). Indeed, mounting evidence in ME/CFS patients implicates a significant role for immunological abnormalities that are thought to contribute to disease progression and/or maintenance of the chronic symptomatic state (Fletcher et al., 2010;Brenu et al., 2012;Curriu et al., 2013;Brenu et al., 2014;Mensah et al., 2017). Studies of the immune system of ME/CFS subjects have revealed many abnormalities, including disruptions in the number and function of T cell subsets, B cell and natural killer (NK) cells (Fletcher et al., 2010;Brenu et al., 2012;Curriu et al., 2013;Brenu et al., 2014); changes in T-cell or innate cell cytokine secretion (Torres-Harding et al., 2008;Broderick et al., 2010;Bansal et al., 2012); changes in humoral immunity (Prinsen et al., 2012) and inflammatory immune signaling (Aspler et al., 2008); and higher frequencies of various autoantibodies (Ortega-Hernandez and Shoenfeld, 2009). In particular, T cells are responsible for orchestrating and modulating an optimal immune response, either through their effector or regulatory functions. Thus, perturbations in T cell subsets or in effector or regulatory functions during ME/CFS (Lorusso et al., 2009;Rivas et al., 2018) can result in an immune disruption or unwanted immune responses.

Here we found perturbations in CD8+ T cells, NK cells, Treg cell frequencies and effector functions of Th17 and MAIT cells in ME/CFS patients. Some of the immune parameter differences, such as CD8+ effector memory T cells and NK cells, were relatively different in younger patients, but not in patients older than 50 years, when compared to healthy controls. In addition, using significant immune features and a machine learning algorithm, we could identify ME/CFS patients among healthy controls with very high sensitivity and specificity. Together, our findings reveal multiple chronic T cell dysfunctions in ME/CFS, suggesting a link to chronic infections or a disruption of the microbiota.

Materials and Methods

Participants

All subjects were recruited at Bateman Horne Center, Salt Lake City, UT, based on were who met the 1994 CDC Fukuda (Fukuda et al., 1994) and/or Canadian consensus criteria for ME/CFS

85 (Carruthers, 2007). Healthy controls were frequency-matched to cases on age, sex, race/ethnicity,
86 geographic/clinical site and season of sampling. Patients or controls taking antibiotics or had any
87 infections in the prior months; taking any immunomodulatory medications were excluded from
88 the study. The study was approved by Western IRB (Protocol number 20151965) and written
89 informed consent and verbal assent when appropriate were obtained from all participants in this
90 study. We enrolled a total of 198 ME/CFS patients and 91 healthy controls. Subject
91 characteristics are shown in supplementary Table 1.

92

93 **PBMC isolation and preservation**

94

95 Healthy and patient blood samples are obtained from Bateman Horne Center, Salt Lake City, UT
96 and approved by Western IRB. Heparinized blood samples were shipped overnight at room
97 temperature. Peripheral blood mononuclear cells (PBMC) were then isolated using Ficoll-paque
98 plus (GE Health care) and cryopreserved in liquid nitrogen.

99

100 **Surface and intracellular staining and flow cytometry analysis**

101

102 After thawing, PBMCs were counted and divided into 2 parts, 1 part for day 0 surface staining,
103 and the other part cultured in complete RPMI 1640 medium (RPMI plus 10% Fetal Bovine
104 Serum (FBS, Atlanta Biologicals) and 1% penicillin/streptomycin (Corning Cellgro)
105 supplemented with IL-2+IL15 (20ng/ml) for Treg subsets day 1 surface and transcription factors
106 staining, IL-7 (20ng/ml) for day 1 and day 6 intracellular cytokine staining and a combination of
107 cytokines (20ng/ml IL-12, 20ng/ml IL-15, and 40ng/ml IL-18) for day 1 intracellular cytokine
108 staining (IL-12 from R&D, IL-7 and IL-15 from Biolegend).

109

110 Surface staining was performed in staining buffer containing PBS + 2% FBS for 30 minutes at
111 4°C. When staining for chemokine receptors the incubation was done at room temperature.
112 Antibodies used in the surface staining are CD3 (UCHT1 clone, Alexa Fluor 532, eBioscience),
113 CD4 (OKT4 clone, Brilliant Violet 510), CD8 (RPA-T8 clone, Pacific Blue or Brilliant Violet
114 570), CD19 (HIB19 clone, Brilliant Violet 510), CD45RO (UCHL1 clone, Brilliant Violet 711,
115 APC/Cy7, or Brilliant Violet 570), CCR7 (G043H7 clone, Alexa Fluor 488), 2B4 (C1.7 clone,
116 PerCP/Cy5.5), CD14 (HCD14 clone, Alexa Fluor 700), CD27 (O323 clone, PE/Cy7, Brilliant
117 Violet 605, or Alexa Fluor 647), CCR6 (G034E3 clone, Brilliant Violet 605), CD161 (HP-3G10,
118 Brilliant Violet 421), Vα7.2 (3C10 clone, PE) (all from Biolegend).

119

120 For intracellular cytokine staining, cells were stimulated with PMA (40ng/ml for overnight
121 cultured cells and 20ng/ml for 6 days cultured cells) and Ionomycin (500ng/ml) (both from
122 Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences) for 4 hours at 37°C. For cytokine
123 secretion after stimulation with IL-12+IL-15+IL-18+, GolgiStop was added to the culture on day
124 1 for 4 hours. Stimulated or unstimulated cells were collected, stained with surface markers
125 including CD3, CD4, CD8, CD161, Vα7.2, CD45RO, CCR6, and CD27 (Biolegend) followed
126 by one wash with PBS (Phosphate buffer Saline) and staining with fixable viability dye
127 (eBioscience). After surface staining, cells were fixed and permeabilized using
128 fixation/permeabilization buffers (eBioscience) according to the manufacturer's instruction.
129 Permeabilized cells were then stained for intracellular IFNγ (4S.B3 clone, APC/Cy7), TNFα
130 (Mab11 clone, PE/Dazze 594), GranzymeA (CB9 clone, Alexa Fluor 647, Alexa Fluor 488), IL-

131 17A (BL168 clone, Alexa Fluor 488, Brilliant Violet 421), Foxp3 (259D clone, PE), and Helios
132 (22F6 clone, Alexa Fluor 488) (all from Biolegend). Flow cytometry analysis was performed on
133 SP6800 spectral cell analyzer (Sony Biotechnology) and analyzed using FlowJo (Tree Star).

134

135 **Machine learning and statistical analysis**

136

137 All statistical analyses were performed using GraphPad Prism V8 software. Continuous variable
138 datasets were analyzed by Mann-Whitney U test for non-parametric datasets when comparing
139 clinical groups, and exact P values are reported. Spearman p was used to determine the
140 relationship existing between two sets of data between non-parametric datasets.

141

142 The algorithms for identifying significantly different features and the RF classifier were
143 implemented in Python 3.6.8 using Jupyter Notebook 5.0.0. The RF classifier was performed
144 with different numbers of features of $k = 65, 40, 10$. A training set with 231 samples (80% of
145 total samples) was selected and the remaining data corresponding to 58 samples (20% of total
146 samples) was left as the test set. Missing values in the training and test sets were replaced by
147 corresponding median value in the training set. The RF classifier was implemented using a 3-
148 fold (stratified) cross validation and was trained using all 65 immune profile features, the 40
149 significantly different features, the top 10 significantly different features and the top 10 features
150 among the 40 significantly different features that received the highest importance score.

151

152 There are several metrics to evaluate the performance of a classifier. Sensitivity represents the
153 proportion of patients who were correctly identified as patients and specificity represents the
154 proportion of healthy controls who were correctly identified as healthy. If patients are denoted by
155 "positives" and healthy controls by "negatives", then sensitivity and specificity are calculated as:

156

157 $$\text{sensitivity} = \frac{\text{Number of true positives}}{\text{Number of positives}} \text{ and specificity} = \frac{\text{Number of true negatives}}{\text{Number of negatives}}$$

158

159 where "true positives" refer to patients who were correctly identified as patients and "true
160 negatives" refer to healthy controls who were correctly identified as healthy.
161 Accuracy is a metric which shows the fraction of predictions that our classifier predicted
162 correctly. Accuracy is calculated in terms of true positives and true negatives as:

163

$$\text{Accuracy} = \frac{\text{Number of true positives} + \text{Number of true negatives}}{\text{Total number of predictions}}$$

164

165 Positive (negative) predictive values are the proportion of positives (negatives) that are correctly
166 identified as positives (negatives which are calculated as:

167

$$\text{Positive predictive value} = \frac{\text{Number of true positives}}{\text{Number of true positives} + \text{Number of false positives}}$$

168

$$\text{Negative predictive value} = \frac{\text{Number of true negatives}}{\text{Number of true negatives} + \text{Number of false negatives}}$$

169

170 The F_1 score measures the accuracy of test by calculating the (harmonic) mean of the sensitivity
171 (recall) and positive predictive value (precision). The F_1 score is defined as:
172

$$F_1 = 2 \times \frac{\text{precision} \times \text{recall}}{\text{precision} + \text{recall}}$$

173
174

175 **Results**

176

177 **Changes in T cells subsets in ME/CFS patient blood**

178

179 To determine phenotypic and functional changes in immune cell subsets from ME/CFS patients,
180 we developed several staining panels for flow cytometry analysis and performed immune
181 profiling of 198 ME/CFS patients and 91 age- and sex- matched healthy controls (Supplemental
182 Table 1). We first analyzed the main immune subsets in peripheral blood mononuclear cells
183 (PBMCs), namely T cells, B cells, NK cells, and monocytes (Figure 1a, 1b). There was no
184 significant difference in the percentage of overall monocytes (p=0.9), B cells (p=0.9) or T cells
185 (p=0.1) (Fig. 1a, 1b), but the frequency of NK cells within lymphocytes was greatly reduced
186 (p=0.0005) in ME/CFS compared to healthy controls (Fig. 1b). Within T cells, we observed that
187 CD4+ T cell frequency was higher (p=0.0193) and correspondingly, CD8+ T cells were lower
188 (p=0.0052) in ME/CFS subjects, and that this was reflected as a higher CD4 to CD8 ratio
189 (p=0.0078) in patients (Fig. 1c). There was no difference in CD4- CD8- (double negative; DN)
190 T cells (p=0.9) between controls and ME/CFS patients (data not shown).

191

192 It is well established that CD4 to CD8 ratio changes are associated with aging (Yan et al.,
193 2001;Yan et al., 2010;Serrano-Villar et al., 2014;Adriaensen et al., 2015). Indeed, CD4+ and
194 CD8+ T cell frequencies and the CD4 to CD8 ratio correlated with age in both healthy controls
195 (r_s=0.4902, -0.4649, and 0.4794 respectively) and in ME/CFS subjects (r_s= 0.4531, -0.4305, and
196 0.4403, respectively) (Fig. 1d). Age also showed a significant correlation with DN T cells for
197 controls (r_s=-0.4384), but not for ME/CFS patients (r_s=-0.2235) (Fig. 1d). Interestingly, when
198 we subdivided ME/CFS patients around their median age, as younger or older than 50 years of
199 age, the difference in the percentage of CD8+ T cells and the CD4:CD8 ratio remained
200 significant only for ME/CFS subjects younger than 50 years (p=0.0082 and 0.0131,
201 respectively), but not for those older than 50 years (p=0.6 and 0.6, respectively) (Fig. 1e). Age
202 also showed a significant correlation with NK cells only in ME/CFS patients (r_s=0.3531), but not
203 in healthy controls (r_s=0.02794) (Fig. 1f). This change in NK cell frequency was also only seen
204 in ME/CFS subjects younger than 50 years (p<0.0001) (Fig. 1g).

205

206 We next divided CD4+ and CD8+ T cells into naïve and memory subsets as part of their
207 differentiation states, based on their functional and phenotypic features (Sallusto et al., 2004). To
208 determine the proportion of these subsets in ME/CFS patients, we used well-established
209 CD45RO and CCR7 cell surface molecules as markers for both CD4+ and CD8+ T cell subsets
210 (Fig. 2a). Within CD4+ T cells, there was no significant difference between ME/CFS patients
211 and healthy controls for CD45RO-CCR7+ (naïve; N) (p=0.5), CD45RO+CCR7+ (central
212 memory; CM) (p=0.7), CD45RO+CCR7- (effector memory; EM) (p=0.2), or CD45RO-CCR7-
213 (effector memory RA; EMRA) (p=0.06) subsets (Fig. 2b). There was also no difference in CD8+

214   N (p=0.4), CM (p=0.1), or EMRA (0.0509) populations, however, the CD8+ EM T cell subset
215   was greatly increased as a proportion of CD8+ T cells (p=0.0001) in ME/CFS patients (Fig. 2c).
216
217   The frequencies of N, CM, EM, and EMRA populations within CD8+ T cells correlated with age
218   for both healthy controls (r_s=-0.5259, 0.5222, 0.3696, and 0.3602 respectively) and ME/CFS
219   patients (r_s=-0.6162, 0.3756, 0.3814, and 0.5172 respectively) (Fig. 2d), and this was also seen
220   in CD4+ subsets (Supplemental Fig. 1a). However, there was no significant difference between
221   ME/CFS patients and healthy controls for CD8+ N or CM T cells for subjects who were younger
222   or older than 50 years (p=0.8 and 0.07, respectively) (Fig. 2d) or for CD4+ N, CM or EM subsets
223   in different age groups of patients and controls (Supplemental Fig. 1b). Interestingly, the CD8+
224   EM subset difference between ME/CFS patients and healthy controls was restricted to subjects
225   younger than 50 years (p=0.0027) (Fig. 2e). CD8+ and CD4+ EMRA subsets were also only
226   significantly lower in ME/CFS patients who were younger than 50 years of age (p=<0.0001 and
227   p<0.0156 respectively) (Fig. 2e and Supplemental Fig. 1b).
228
229   **Changes in Th17 cell frequency and function in ME/CFS disease**
230
231   We hypothesized that ME/CFS patients may also have disruptions within effector T cell subsets
232   resident in mucosal tissues such as Th17 cells, which respond to bacterial infections or
233   microbiota and are also linked to autoimmune diseases (Milner et al., 2010;Pandiyan et al.,
234   2019). To identify Th17 cells we first used CD3, CD4, CD45RO, and CCR6 expression (Fig.
235   3a), as almost all of this subset has a memory phenotype and also expresses the chemokine
236   receptor CCR6 (Romagnani et al., 2009). In order to analyze the cytokine secretion from T cells,
237   we thawed frozen aliquots of PBMC and cultured one day in IL-7 (d1) to ensure cells recovered
238   from thawing and any dying cells could be clearly identified. We then activated the cells with
239   PMA and Ionomycin as described in methods. The cells were then stained intracellularly for IL-
240   17 and IFNγ and gated on cell surface expression based on CD4+CD45RO+CCR6+ and
241   CD4+CD45RO+CCR6- cells (Fig. 3b), as previously described (Wan et al., 2011). Within the
242   CD4+CD45RO+ (memory T cell) population, the frequency of IL-17+ (p=0.0378), IFNγ+
243   (p=0.0231), and IL-17+IFNγ+ (p=0.0378) secreting cells was significantly reduced in ME/CFS
244   compared to healthy subjects (Fig. 3c).
245
246   Previously we have shown that a portion of Th17 cells are poised to produce IL-17 or IL-22 only
247   after priming with γc-cytokines (namely IL-2, IL-15 or IL-7) in culture, which reveal their full
248   potential of their IL-17 secretion (Wan et al., 2011). Accordingly, we cultured PBMC from
249   ME/CFS patients and control subjects for 6 days (d6) in IL-7 to prime Th17 cells for IL-17
250   secretion, as previously described (Wan et al., 2011). PBMC were then stimulated using PMA
251   and Ionomycin, and expression of cytokines within T cell subsets was determined. In this assay,
252   T cells from ME/CFS patients expressed profoundly lower total IL-17+ (p<0.0001), IFNγ
253   (p<0.0001), IL-17+IFNγ+ (p<0.0001), and IL-17+IFNγ- (p<0.0001) cells (Fig. 3d), revealing a
254   major dysfunction of Th17 cells in patients.
255
256   After 6 days in culture with IL-7, the proportion of IL-17 and IFNγ secreting cells within
257   CD4+CD45RO+ memory population of healthy controls did not correlate with age for either IL-
258   17+, IFNγ+, IL-17+IFNγ+, or IL-17+IFNγ- subsets (r_s=-0.2379, -0.2929, -0.2413, and -0.2719
259   respectively). For ME/CFS patients, age also did not correlate with IFNγ+ expressing cells (r_s=-

260   0.2929), but IL-17+, IL-17+IFNγ+, and IL-17+IFNγ- subsets showed a significant correlation
261   (r_s=-0.4073, -0.3952, and -0.3784, respectively) (Fig. 3e). When patients were broken down into
262   groups of subjects younger and older than 50 years, significant differences were observed in IL-
263   17+, IFNγ+, IL-17+IFNγ+, and IL-17+IFNγ- subsets between controls and ME/CFS subjects
264   younger than 50 years (p=0.0017, 0.0018, 0.0009, and 0.0009, respectively), as well as among
265   the older than 50 years groups (p=0.0002, 0.0047, 0.0008, and 0.0001, respectively) (Fig. 3f).

267   To further investigate the disruption in the Th17 cell subset, we compared the frequency of
268   CD4+CD45RO+CCR6+ cells between controls and ME/CFS patients. In contrast to IL-17
269   expression, we found that CCR6+ cells were significantly higher in ME/CFS patients
270   (p=0.0009). However, after 6 days in IL-7, there was no difference between the subject groups
271   (p=0.2), even though the average frequency was higher in both groups (Fig. 4a). CCR6+ cell
272   frequency within memory CD4 T cells correlated with subject age for healthy controls
273   (r_s=0.3206), but not for ME/CFS patients (r_s=0.2071). When patients were grouped as younger
274   and older than 50 years of age, a significant difference was seen for the proportion of CCR6+
275   cells only in subjects younger than 50 years (p=0.0002) (Fig. 4b).

277   Remarkably, ME/CFS subjects, compared to controls, displayed lower expression of IL-17+
278   (p=0.0035), IL-17+IFNγ+ (p=0.0055), and IL-17+IFNγ- (p=0.0084), but not total IFNγ+ (p=0.3),
279   within the CD4+CD45RO+CCR6+ T cells (Fig. 4c). After 6 days in culture in IL-7, the
280   differences further increased and were seen in all cytokine-secreting cells, as a proportion of
281   CD4+CD45RO+CCR6+ T cells, for IL-17+ (p<0.0001), IFNγ+ (p=0.0010), IL-17+IFNγ+
282   (p<0.0001), and IL-17+IFNγ- (p<0.0001) cells (Fig. 4d).

284   We next determined the ratio between the CCR6+ T cells to CD4+ memory T cells expressing
285   IL-17 or IFNγ. Indeed, the ratio of CCR6+ cells to IL-17+ (p<0.0001) and to IFNγ+ (p<0.0001)
286   CD4+ memory T cells were significant in ME/CFS patients compared to healthy controls (Fig.
287   4e). These ratios between CCR6+ cells and cytokines produced by CD4+ cells also remained
288   higher in ME/CFS subjects after d6 in IL-7, for CCR6+ to IL-17+ cell ratio (p=0.0015), but were
289   only marginally different for CCR6+ to IFNγ+ cell ratio (p=0.0366) (Fig. 4f).

291   We have previously shown that CD161 within the CD4+CD45RO+CCR6+ T cells can further
292   divide these cells into subsets with differences in IL-17 and IFNγ secretion (Wan et al., 2011).
293   As such, we further divided CCR6+ cells based on CD161 expression (Fig. 5a). The proportion
294   of CD161+ cells within the CCR6+ subset was only slightly different in ME/CFS compared to
295   controls (p=0.0439) (Fig. 5a). We then analyzed IL-17 and IFNγ expression within the CD161+
296   and CD161- subsets of CD4+ CD45RO+ CCR6+ cells after 6 days in culture (Fig. 5b). Within
297   the CD4+CCR6+CD161+ cells, there was a significant difference in expression of IL-17+, IL-
298   17+IFNγ+, and IL-17+IFNγ- cells between ME/CFS and controls (p<0.0001 for all), but not for
299   IL-17-IFNγ+ cells (p=0.06) (Fig. 5c). CD161- cells within the CD4+CD45RO+CCR6+ subset
300   also displayed lower IL-17+, IL-17+IFNγ+, IL-17+IFNγ-, and IL-17-IFNγ+ in ME/CFS subjects
301   compared to healthy controls (p=<0.0001, <0.0001, 0.0001, and 0.0042, respectively), (Fig. 5d).

303   In CD4+ memory T cells, in addition to IL-17 expression, we also determined the frequency of T
304   cells that were either expressing IFNγ (IFNγ+IL-4-) or IL-4 (IFNγ-IL-4+) only, which
305   respectively define Th1 and Th2 T cell subsets (Fig. 5e). We found that the proportion of

306   IFNγ+IL-4- and IFNγ-IL-4+ within CD4+ memory T cells was significantly lower (p=0.0157
307   and p<0.0001 respectively) in ME/CFS subjects (Fig. 5e). However, the ratio of Th1 (IFNγ+IL-
308   4-) to Th2 (IFNγ-IL-4+) was higher in ME/CFS patients compared to the control group
309   (p=0.0196) (Fig. 5b), suggesting an imbalance of Th1 to Th2 cells. Together, these findings
310   highlight major functional perturbations within the CD4+ T cell subset in the ME/CFS patient
311   cohort.

312

313   **Changes in Frequency of MAIT cells in ME/CFS**

314

315   Mucosal-associated invariant T (MAIT) cells are a subset of the non-classical T cell population
316   and defined by an invariant T cell receptor that is triggered by riboflavin metabolites produced
317   by bacteria, including commensal microbiota (Tastan et al., 2018;Godfrey et al., 2019). Similar
318   to the Th17 subset, we hypothesized that dysbiosis in the gut microbiome or prior bacterial
319   infections may result in changes in MAIT cell frequencies or function. To identify MAIT cells in
320   PBMC, we used Vα7.2 and CD161 surface molecules as previously described (Khaitan et al.,
321   2016;Tastan et al., 2018). We then determined the frequency of MAIT cells within CD4+, CD8+
322   and CD4-CD8- (double negative or DN) T cell compartments in ME/CFS patients and healthy
323   controls (Fig. 6a). There was no significant difference between patients and controls for CD4+
324   (p=0.7), CD8+ (p=0.7), or double negative (DN) MAIT cells (p=0.2) as a proportion of the
325   CD4+, CD8+ and DN T cells respectively (Fig. 6b). However, CD4+ and CD8+ MAIT cell
326   frequencies in PBMC after 6-day culture in IL-7 showed a significant difference (p=0.0250 and
327   p=0.0221 respectively) between ME/CFS patients and controls, but DN MAIT cell frequency did
328   not change between ME/CFS and control samples after 6 days culture (p=0.3) (Fig. 6c). When
329   the ratio of MAIT cell frequency at day 0 (d0) vs day 6 after IL-7 culture (d6) was assessed, we
330   found that the frequency of CD8+ MAIT cells in ME/CFS PBMC was greatly reduced after 6
331   days of culture compared to d0 levels (p=0.0008), but there was no significant difference seen
332   for CD4+ (p=0.06) or for DN MAIT cells (p=0.8) between ME/CFS patients and controls (Fig.
333   6d). Corollary to this finding, the ratio of CD8+ MAIT to DN MAIT cells in ME/CFS patients
334   and controls was only slightly significant at d0 (p=0.0364), but became highly significant after 6
335   days in culture with IL-7 (p<0.0001) (Fig. 6e). Together, these findings suggest that CD8+
336   MAIT cells from ME/CFS subjects survived less in in vitro culture with IL-7.

337

338   Because CD27 expression on MAIT cells could indicate a recently activated or differentiated
339   subset, similar to other CD8 T cells (Dolfi and Katsikis, 2007;Grant et al., 2017), we evaluated
340   CD27 expression in MAIT subsets (Fig. 6f). We found that ME/CFS patients had a significant
341   difference where there were higher CD45RO+CD27- cells compared to the control group
342   (p=0.0045), but interestingly, this difference was not observed within DN MAIT cells (p=0.7)
343   (Fig. 6g). The d0 to d6 CD8+ MAIT cell ratio also displayed a slight positive correlation with the
344   frequency of CD27-CD8+ MAIT cells in patients (r_s=0.2920), but not in healthy controls
345   (r_s=0.2519). In contrast, CD27- DN MAIT cells did not correlate with the d0 to d6 cell
346   frequency ratio for either controls (r_s=0.1597), or ME/CFS patients (r_s=0.1016) (Fig. 6h).

347

348   We then asked to what extent MAIT cells were functionally different between ME/CFS patients
349   and controls. For this approach we first stimulated the PBMC with a cocktail of three cytokines,
350   namely IL-12+IL-15+IL18, since this combination has been uniquely shown to induce
351   expression of IFNγ from MAIT cells (Ussher et al., 2014;Salou et al., 2017). Accordingly, IFNγ

352　along with Granzyme A expression was used to evaluate the response of CD8+ MAIT and CD8+
353　non-MAIT cells in PBMC to stimulation with IL-12+IL-15+IL18 cocktail (Fig. 7a). ME/CFS
354　patient PBMC stimulated with the cytokine cocktail showed much lower IFNγ+ MAIT cells
355　(p=<0.0001), but induction of IFNγ+ from non-MAIT CD8+ T cells was comparable (p=0.1) to
356　healthy subjects (Fig. 7b). Granzyme A expressing MAIT cells were also much higher in
357　ME/CFS subjects (p<0.0001), but non-MAIT cells expressing Granzyme A were not different
358　(p=0.8) between controls and patients (Fig. 7c). In addition, CD27-CD8+ MAIT cells and IFNγ+
359　MAIT cells upon cytokine stimulation were negatively correlated in ME/CFS patients (r_s=-
360　0.3431) but not in controls (r_s=-0.2112) (Fig. 7d). CD27-CD8+ MAIT cells were not correlated
361　with CD8+ non-MAIT IFNγ+ cells for either healthy controls (r_s=-0.1370) or ME/CFS patients
362　(r_s=-0.09816) (Fig. 7d).

364　Since MAIT cells have also been shown to express IL-17, similar to Th17 cells (Salou et al.,
365　2017), we next sought to determine the production of IL-17 and IFNγ from MAIT cells in
366　response to PMA and Ionomycin stimulation. There was very little to undetectable IL-17
367　expression from MAIT cells after one day in culture (data not shown). However, after 6 days in
368　IL-7, MAIT cells expressing IL-17 were greatly increased upon PMA and Ionomycin
369　stimulation, however, IL-17 remained undetectable in non-MAIT CD8+ T cells (Fig. 7e). This
370　finding suggests that MAIT cells can also undergo priming with cytokines, similar to the classic
371　Th17 cells (Wan et al., 2011) and IL-17 expression mimics tissue-resident MAIT cells
372　(Sobkowiak et al., 2019).

374　In addition, we also determined IFNγ and TNFα secretion from CD8+ MAIT and CD8+ non-
375　MAIT CD45RO+ (memory) T cells after 6 days in culture with IL-7 (Fig. 7f). There was no
376　significant difference in ME/CFS patients for IFNγ+ MAIT cells (p=0.5), but we found highly
377　reduced IL-17+IFNγ+ MAIT cells in ME/CFS patients compared to healthy controls (p=0.0075)
378　(Fig. 7g). The frequency of IFNγ+ secreting cells was also reduced within CD8+ non-MAIT cells
379　(p=0.0057) and within CD8+CD45RO+ memory T cells (p=0.0002), in ME/CFS PBMC cultured
380　for 6 days in IL-7 (Fig. 7h).

382　**Changes in regulatory T (Treg) cells in ME/CFS patients**

384　Regulatory T (Tregs) cells are critical in controlling autoreactive or excessive immune responses.
385　Given the observed perturbance in the effector functions of T cell subsets that suggest chronic
386　immune activation, we hypothesized that there would be a corresponding increase in Tregs in
387　ME/CFS patients. For this experiment, we used Foxp3 and Helios as markers to assess Treg cell
388　frequencies within both naïve and memory CD4+ T cells, as previously described (Mercer et al.,
389　2014) (Fig. 8a). Indeed, frequencies of both naïve Tregs (p=0.0005), and memory Tregs
390　(p=0.0094) were increased in ME/CFS compared to controls (Fig. 8b).

392　When broken down into groups where subjects were younger or older than 50 years, naïve Tregs
393　showed a highly significant difference in ME/CFS patients vs controls in the younger than 50
394　years group (p=0.0083), and a slightly significant difference in ME/CFS patients vs controls in
395　the older than 50 years group (p=0.0209). The difference in memory Tregs was also significant
396　between ME/CFS patients and controls younger than 50 years (p=0.0116), but not when older
397　than 50 years groups were compared (p=0.6) (Fig. 8c). There was no correlation with subject age

398   for ME/CFS patients or controls for naïve Tregs (r_s=-0.02541 and r_s=-0.01592, respectively), or
399   for memory Tregs (r_s=0.2691 and r_s=0.1585, respectively) (Fig. 8d).
400
401   The ratio of Th17 cells to Tregs is an important feature that is perturbed during chronic
402   inflammatory conditions or autoimmune diseases. Therefore, we also determined this ratio in
403   ME/CFS patients vs healthy controls. While the Th17 (CCR6+ IL-17-secreting cells) frequency
404   did not correlate with memory Treg cells in ME/CFS patients (r_s=0.2750) or healthy controls
405   (r_s=-0.08416), remarkably, the ratio of these two related subsets were also highly different
406   between the ME/CFS patients and the healthy controls (p<0.0001) (Fig. 8e)
407
408   **Machine learning analysis to identify predictive immune parameters for ME/CFS**
409
410   Our immune profiling analysis identified many T cell subset parameters that were different in
411   ME/CFS patients vs healthy controls. From the total of 65 immune profile features, 40 features
412   were identified as different at a 5% false discovery rate (Supplemental Table 2). However, while
413   some of these were highly significant, given the high variability and ranges in humans for
414   immune parameters, on their own they would not have clinically relevant specificity and
415   sensitivity to discriminate patients from healthy individuals. Therefore, we decided to use a
416   classifier model using a machine learning algorithm called the random forest (RF) classifier
417   (Wang and Li, 2017).
418
419   The RF classifier or algorithm is an ensemble method that depends on a large number of
420   individual classification trees (Wang and Li, 2017;Huynh-Thu and Geurts, 2019). Each
421   classification tree emits a predicted class and the class with the most votes becomes the model
422   prediction. The individual trees are designed using a randomly selected number of samples
423   (sampling with replacement) and a randomly selected feature set to minimize correlation
424   between trees. A large number of relatively uncorrelated classification trees (models) are
425   combined to provide a robust classification of the individual sample (Aevermann et al., 2018).
426   As such, we implemented an RF model to classify ME/CFS patients and healthy controls using
427   the immune profiling data. The performance of the RF was evaluated using the receiver
428   operating characteristic (ROC) curve, which is created by plotting the true positive rate (TPR)
429   against the false positive rate (FPR). The class prediction probability of a sample can be
430   computed based on the proportion of votes obtained for that call. Given a threshold T for the
431   probability, a sample is classified as an ME/CFS patient if the probability is higher than T and
432   the ROC curve plots TPR against the FPR.
433
434   The area under the ROC curve which is denoted by AUC is equal to the probability that a
435   randomly chosen positive instance will be ranked higher than a randomly chosen negative
436   instance. A perfect classifier will have the maximal area under the curve of 1. The ROC curves
437   of the RF classifier corresponding to 4 subsets of immune profile features are shown in Figure 9.
438   The AUC of the RF classifier using all 65 features is ~0.93, meaning that there is a chance of 93%
439   that the classifier will correctly distinguish between patients and healthy controls (Fig. 9 and
440   Table 1). The 40 significantly different features or top 10 features with the highest importance
441   score among these 40 significantly different immune parameters had slightly lower AUC scores
442   (~0.92 and ~0.88, respectively), whereas the top 10 significantly different features had a lower
443   AUC score (~0.82) (Fig. 9 and Table 1, full list of the features are in supplemental Tables 2, 3).

444
Discussion
446

447 Several studies of the immune system of ME/CFS subjects have revealed disruptions in the
448 number and function of T cell subsets, B cells and natural killer (NK) cells in patient blood
449 (Fletcher et al., 2010;Brenu et al., 2012;Curriu et al., 2013;Brenu et al., 2014). Here we extend
450 these findings and show a highly significant disruption in several key T cell subset frequencies,
451 and importantly, their effector functions. Our findings reveal profound changes in the functional
452 capacity of mucosal associated T cell (MAIT) and Th17 cells, Tregs, and signs of CD8+ T cell
453 and NK cell disruption in a subset of younger subjects. Given that these cell types are involved in
454 regulating viral or bacterial infections or the microbiota, our findings suggest that some of the
455 ME/CFS patients have severe immune perturbations likely triggered by chronic infections or are
456 associated with changes in the microbiome.
457

458 Our observation that both CD8+ T cells and NK cells are proportionately reduced in ME/CFS
459 subjects may also reflect chronic viral infection or persistence (such as CMV or EBV infections)
460 that triggers a chronic activated state of these cells. It is important to note that this difference was
461 predominantly observed in subjects younger than 50 years old, as healthy subjects over the age
462 of 50 also begin to display a similar phenotype as patients and thus were no longer statistically
463 different compared to patient subjects. Indeed, this is consistent with the increase in chronic viral
464 infections seen during normal aging, such as CMV seropositivity (Ellefsen et al., 2002).
465 However, given this distribution, these subset differences alone are unlikely to be the main cause
466 of ME/CFS symptoms as it is apparent there are also multiple and severe changes in the effector
467 functions of other T cell subsets. In addition, the proportion of CMV/EBV infection among
468 healthy controls and ME/CFS patients was not significantly different in either age group (data
469 not shown).
470

471 Another T cell subset that we found to be highly disrupted in ME/CFS subjects was Th17 cells,
472 which are characterized by secretion of IL-17, expression of chemokine receptor CCR6 and
473 transcription factor RORC (Sallusto et al., 2012). Th17 cells play a major role in both immune
474 response to bacterial or fungal infections and in pathogenesis of autoimmune or chronic
475 inflammatory diseases (Tesmer et al., 2008;Sallusto and Lanzavecchia, 2009). We previously
476 found that a significant portion of cells programmed towards the Th17 phenotype do not
477 immediately secrete IL-17 but require a priming stage with IL-7 or IL-15, which we call poised
478 Th17 cells (Wan et al., 2011). Indeed, when we performed an assay by culturing PBMC in IL-7
479 for 6 days before analyzing the IL-17 production from these poised CCR6+ Th17 cells, the
480 differences between ME/CFS patients and controls became profound. However, while there were
481 less Th17 cells producing IL-17 in ME/CFS patients, paradoxically the proportion of CCR6+
482 Th17 cells were significantly higher compared to controls. Together these findings suggest
483 chronic activation of the Th17 subset that potentially induces an "exhausted" state, as when their
484 numbers are increased due to chronic stimulation, they become more dysfunctional. One possible
485 culprit for chronic Th17 cell stimulation could be the changes in the composition of microbiota,
486 or dysbiosis that is caused by shifting balances in beneficial or harmful bacteria species
487 (Omenetti and Pizarro, 2015), as seen during HIV infection (Lujan et al., 2019), and as
488 contributes to autoimmune diseases (Gulden et al., 2015). There is also evidence that Th17 cells
489 can respond to specific microbiota-associated bacteria such as *Prevotella* species (Larsen, 2017).

490   Indeed, several reports have identified microbiome changes in ME/CFS (Shukla et al.,
491   2015;Giloteaux et al., 2016;Navaneetharaja et al., 2016;Nagy-Szakal et al., 2017;Proal and
492   Marshall, 2018).
493
494   Regulatory T cells (Tregs) are tasked with suppressing autoimmune and excessive chronic
495   inflammatory responses (Sakaguchi et al., 2010;Yamaguchi et al., 2011). Our finding that Tregs
496   are increased in ME/CFS patients is consistent with our other findings that there appears to be a
497   chronic activation of major T cell subsets with yet to be identified stimuli. Increased Tregs in
498   patients may reflect the disturbance of the Th17 and Th1/Th2 cytokine balance in ME/CFS
499   patients, suggesting a major disruption in homeostatic maintenance of immune responses.
500   Indeed, for example, the balance between Th17 cells and Tregs is also critical in the regulation of
501   inflammation, especially related to the gut and microbiome (Omenetti and Pizarro,
502   2015;Pandiyan et al., 2019). Remarkably, we found a correlation between Th17 and Treg cells in
503   ME/CFS patients, and found the ratio was even more significantly different in patients compared
504   to controls. These findings reveal potential biomarkers that can be utilized in future clinical
505   interventions to re-balance the microbiome and the mucosal immune responses. In future studies
506   it will also be important to further investigate in more detail the different subsets of Tregs for
507   their suppressive capacity, and as with other subsets, to identify the mechanisms that lead to their
508   increase in ME/CFS patients.
509
510   In our analysis, another T cell subset that was profoundly different between patients and controls,
511   both phenotypically and functionally, was MAIT cells. MAIT cells selectively respond to a
512   broad range of bacteria that possess the biosynthetic pathway for riboflavin metabolism (Gold et
513   al., 2010;Le Bourhis et al., 2010;Kjer-Nielsen et al., 2012;Meierovics et al., 2013). A recent
514   study found significant changes in the frequency of CD8+ MAIT cells in Multiple Sclerosis and
515   severe ME/CFS patients (Cliff et al., 2019). We did not observe significant changes in the
516   frequency of MAIT cells as a percentage of PBMC or T cells, which could be due to fact that this
517   T cell subset is highly variable and has up to a 40-fold difference in range, even among healthy
518   humans (Ben Youssef et al., 2018), and that most of our patient group was not characterized as
519   severe. However, we observed profound differences in MAIT cell functions and differentiated
520   states in ME/CFS subjects, such as reduced production of cytokines (IFNγ, IL-17) or cytotoxic
521   molecule GranzymeA, and an increased proportion of CD27 negative CD8+ MAIT cells, which
522   correlated with their lower survival in 6 day culture with IL-7 in vitro. Given that MAIT cells are
523   specifically activated by a bacteria-produced riboflavin (vitamin B2) metabolite, we reason that
524   this perturbation in MAIT cells can also be associated with differences in the composition of the
525   microbiota of the patients. Indeed, we recently showed that MAIT cells can respond to a variety
526   of microbiome-related bacteria (Tastan et al., 2018) and that they potentially function to tune or
527   sense the microbial ecosystem throughout mucosal tissues. Two recent papers also show that
528   MAIT cell development and expansion is also directly dependent on the microbiome
529   (Constantinides et al., 2019;Legoux et al., 2019;Oh and Unutmaz, 2019). In one paper, the
530   authors show that CD4-CD8- (double negative, or DN) MAIT cells are a functionally distinct
531   subset that migrate to the skin in mice and are potentially involved in tissue repair (Dias et al.,
532   2018;Constantinides et al., 2019;Oh and Unutmaz, 2019). Thus, it is interesting to note that we
533   observed differences in CD8+ but not in DN MAIT cells in ME/CFS subjects, suggesting that
534   these are different lineages or subsets. Taken together, it is conceivable that a disruption in the

microbiome results in chronic activation of MAIT cells and an exhausted state in ME/CFS patients, which is similar to what has been seen with the Th17 subset (Cliff et al., 2019).

Finally, using immune parameters as features, our machine learning classifier was able to identify the ME/CFS patients at a high sensitivity and accuracy when using all 65 features, all 40 significantly different features and the 10 features among the 40 significantly different features that had the highest importance score. For all cases, we observed a higher value of sensitivity than specificity, indicating that the proportion of patients identified as ME/CFS patients is higher than healthy controls who are correctly identified as healthy. One reason for this could be related to features such as age, which causes the older individuals' immune profiles to become more similar to those of ME/CFS patients, and hence the RF classifier categorizes healthy controls as patients. Currently, the diagnosis of ME/CFS is based on clinical symptoms alone and runs the potential for false positives and negatives. A classifier based on immune profiles might be an objective solution to better diagnose this clinical problem, but additional testing on larger and more diverse clinical cohorts will be required to assess the potential clinical utility of such an approach.

In conclusion, our findings open an exciting path to develop a set of biomarkers that can be utilized to aid in both diagnosis and in stratifying the patient population for targeted or precision medicine therapeutics.

Data Availability

The source flow cytometry data for each subject underlying Figs. 1-8 and supplemental figures and tables will be provided upon reasonable request.

Ethics Statement

This study was carried out in accordance with the recommendations of the Jackson Laboratory human subject ethics committee, and IRB protocol approval by Western Institutional Review board. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Author Contribution

E.K. and C.L.G. contributed equally. E.K. processed blood samples, performed the majority of the experiments and analysis, and helped with the preparation of the manuscript; C.L.G. performed the statistical analyses and helped with the preparation of the manuscript; V.R. and J.G. performed machine learning analysis; P.N.G provided advice and feedback for machine learning analysis and interpretation; S.R., M.H. and L.P helped with processing blood samples, performed experiments and analysis; L.K., designed and quality checked all the immune panels; L.B. and S.D.V. recruited all subjects and clinically diagnosed ME/CFS patients; D.U. conceived and supervised the study and wrote the manuscript. All authors reviewed and approved the final draft of the manuscript.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

Abbreviations

ME/CFS: myalgic encephalomyelitis/chronic fatigue syndrome
PBMC: peripheral blood mononuclear cells
DN: double negative; CD4- CD8-
NK: natural killer
MAIT: mucosal-associated invariant T
Treg: T regulatory cells
N: naive
CM: central memory
EM: effector memory
EMRA: effector memory RA
PMA: phorbol 12-myristate 13-acetate
p: probability value
r_s: Spearman's Rank Correlation Coefficient
K: number of folds in cross validation
k: number of features
RF: random forest
TPR: true positive rate
FPR: false positive rate
ROC: receiver operating characteristic
AUC: area under the curve
F1: F score; measure of a test's accuracy
EBV: Epstein-Barr virus
CMV: cytomegalovirus
HIV: human immunodeficiency virus

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829 **Figure Legends**
830

831 **Figure 1. Frequency of CD4+ and CD8+ T cell subsets in ME/CFS patients and healthy**
832 **controls.** Analysis of frequency of main immune subsets in PBMC by flow cytometry was
833 performed after staining and gating on (a) CD14+ (Monocytes) and CD19+ (B cells), and (b)
834 CD3+ (T cells) and CD3-2B4+ (NK cells), and proportion of each subset frequency as a portion
835 of PBMC are shown for each subject (right panels). (c) Frequencies of CD4+ and CD8+ T cells
836 and their ratio were analyzed within CD3+ T cell gates. (d) Correlation of T cell subsets with age
837 in ME/CFS patients and controls. (e) CD8+ T cell and CD4 to CD8 T cell ratio distribution in
838 ages older and younger than 50 years in ME/CFS and controls, (f) Correlation of NK cells with
839 age in ME/CFS patients and controls, (g) NK cell ratio distribution in groups based on ages older
840 and younger than 50 years. Data from healthy controls (Healthy, n = 90) and ME/CFS patients
841 (ME/CFS, n = 186) for a (left), from Healthy (n = 91) and ME/CFS (n = 190) for a (right), b
842 (left), c, d, and e, from Healthy (n = 91) and ME/CFS (n = 189) for b (right), f, and g, and were
843 compared by Mann-Whitney U test for non-parametric data, with exact p values, average (AVG)
844 and median (MED) values are shown. Correlations of data were performed using nonparametric
845 Spearman correlation, with exact r_s and p value shown.

846

847 **Supplemental Figure 1.** (a) The frequency of each subset was correlated to subject age for
848 CD4+ T cells, with clinical groups compared by nonparametric Spearman correlation, with exact
849 r_s and p-value shown. (b) Analysis of CD4+ T cell subset frequencies in healthy control and
850 ME/CFS patients that have been divided into two groups based on ages older and younger than
851 50 years. Data from healthy controls (Healthy, n = 91) and ME/CFS patients (ME/CFS, n = 190)
852 for a and b, and clinical groups were compared by Mann-Whitney test for non-parametric data,
853 with exact p values shown.

854

855 **Figure 2. Frequencies of Naïve and memory T cell subset in ME/CFS patients.** (a) T cell
856 subsets were analyzed in PBMC with CD45RO and CCR7 expression after gating on
857 CD3+CD4+ (left) and CD3+CD8+ T cell subsets (right), which were then subdivided into
858 CD45RO-CCR7+ or naïve (N), CD45RO+CCR7+ or central memory (CM), CD45RO+CCR7-
859 or effector memory (EM), and CD45RO-CCR7- effector memory RA (EMRA) subsets as
860 shown. (b) Proportions of naïve (N), central memory (CM), effector memory (EM) and effector
861 memory RA (EMRA) T cell subsets were analyzed within CD3+CD4+ T cells and (c)
862 CD3+CD8+ T cells in ME/CFS and healthy subjects. (d) The frequency of each subset was
863 correlated to subject age for CD8+ T cells, by nonparametric Spearman correlation, with exact r_s
864 and p-value shown. (e) Analysis of CD8+ T cell subset frequencies in controls and ME/CFS
865 patients that have been divided into two groups based on ages older and younger than 50 years.
866 Data from healthy controls (Healthy, n = 91) and ME/CFS patients (ME/CFS, n = 190) for b-e,
867 and groups were compared by Mann-Whitney test for non-parametric data, with exact p values
868 shown, average (AVG) and median (MED) values are also shown. Correlations of data were
869 performed using nonparametric Spearman correlation, with exact r_s and p value shown.

870

871 **Figure 3. Analysis of Th17 cell frequency and function in ME/CFS subjects.** PBMC purified
872 from patient or control blood were cultured for one day (d1) in IL-7 and then stimulated and
873 stained with specific antibodies shown for flow analysis, as described in methods. (a)
874 CD3+CD4+ cells were gated and the proportion of CD45RO+ and CCR6+ or CCR6- cells

875 analyzed. (b) CD4+ memory (CD45RO+) T cells expressing IFNγ and/or IL-17 or after gating
876 into CCR6+ and CCR6- T cells. (c) The frequency of IL-17 and/or IFNγ expression in
877 CD4+CD45RO+ memory T cells in ME/CFS patient or control PBMC. (d) Same analysis was
878 performed in PBMC after 6-day (d6) culture in IL-7. (e) Correlation of CD4+CD45RO+ memory
879 T cells secreting IL-17 and/or IFNγ with subject age. Groups compared by nonparametric
880 Spearman correlation, with exact r_s and p-value shown. (f) Analysis of CD45RO+ memory IL-
881 17 and/or IFNγ producing cells in control and ME/CFS patients divided into two groups based on
882 ages older and younger than 50 years. Data from healthy controls (Healthy, n = 80) and ME/CFS
883 patients (ME/CFS, n = 198) for c, from Healthy (n = 90) and ME/CFS (n = 195) for d, e, and f,
884 and groups were compared by Mann-Whitney test for non-parametric data, with exact p-values,
885 average (AVG) and median (MED) values shown. Correlations of data were performed using
886 nonparametric Spearman correlation, with exact r_s and p value shown.

888 **Figure 4. Disruption of Th17 cells in ME/CFS patients.** (a) Proportion of CCR6+ T cells in
889 memory CD4+ cells after day 1 (d1) or day 6 (d6) in culture in IL-7. (b) The frequency of
890 CCR6+ T cells in memory CD4+ cells after day-1 culture correlated to subject age. Groups
891 compared by nonparametric Spearman correlation, with exact r_s and p-value shown. Analysis of
892 CCR6+ T cells in memory CD4+ cells after day-1 culture in healthy control and ME/CFS
893 patients divided into two age groups, (c) IL-17 and IFNγ expression in CD4+CCR6+CD45RO+
894 T cells in PBMC culture in IL-7 for 1 day or (d) for 6 days, post activation as described in
895 methods. (e) Ratio of CD4+CCR6+ cells to IL-17+ or total IFNγ+ CD4+ memory cells
896 calculated after cells after day-1 culture or (f) after 6 days in culture with IL-7. Data from healthy
897 controls (Healthy, n = 81) and ME/CFS patients (ME/CFS, n = 198) for a (left), from Healthy (n
898 = 90) and ME/CFS (n = 195) for a (right), from Healthy (n = 80) and ME/CFS (n = 197) for b,
899 from Healthy (n = 80) and ME/CFS (n = 198) for c and e, from Healthy (n = 90) and ME/CFS (n
900 = 196) for d, from Healthy (n = 90) and ME/CFS (n = 195) for f, and groups were compared by
901 Mann-Whitney test for non-parametric data, with exact p values shown. Average (AVG) and
902 median (MED) are also shown. Correlations of data were performed using nonparametric
903 Spearman correlation, with exact r_s and p value shown.

905 **Figure 5. Disruption of Th17 cells.** PBMC from ME/CFS patients and healthy controls were
906 cultured in IL-7 for 6 days and stimulated with PMA/ionomycin for 4 hours as described in
907 methods. (a) Live CD4+ T cells were gated on different memory subsets based on CD161
908 expression (left) and the proportion of CD161+ cells within CD4+CD45RO+CCR6+ subset is
909 shown for each subject (right). (b) Intracellular expression of IL-17 and IFNγ within
910 CD4+CCR6+CD161+ and CD4+CCR6+CD161- T cell subsets. (c) The frequencies of IL-17 and
911 IFNγ expressing cells within CD4+CD45RO+CCR6+CD161+ and (d)
912 CD4+CD45RO+CCR6+CD161- cells were calculated and shown for individual study
913 participants. (e) Analysis of IFNy+IL4- (Th1 cells) and IFNγ- IL4+ (Th2 cells) in memory CD4+
914 cells after day 1 (d1) in culture in IL-7, and ratio of Th1 to Th2 cells. Data from healthy controls
915 (n = 90) and ME/CFS patients (n = 196) for a (right), from Healthy (n = 87) and ME/CFS (n =
916 191) for c and d, from Healthy (n = 90) and ME/CFS (n = 198) for e, and groups were compared
917 by Mann-Whitney test for non-parametric data, with exact p-values and average (AVG) and
918 median (MED) values are shown. Correlations of data were performed using nonparametric
919 Spearman correlation, with exact r_s and p value shown.

Figure 6. Perturbation of Mucosal Associated Invariant T (MAIT) cell subsets in ME/CFS PBMC. (a) MAIT cell subset frequencies were identified based on the co-expression of CD161 and Vα7.2, after gating within CD4+, CD8+ and CD4-CD8- (DN) T cells (b) Analysis of the proportion of MAIT cells in each of these T cell subsets on day 0 (d0) and (c) 6 days (d6) in culture with IL-7. (d) Ratio of day 0 to day 6 MAIT cells was calculated for individual study participants. (e) The ratio of CD8+ MAIT to DN MAIT cells was calculated for day 0 (left) and day 6 (right). (f) Surface expression of CD27 was determined after gating for CD8+ MAIT and DN MAIT cell subsets as shown. (g) Analysis of the proportion of CD45RO+CD27- within CD8+ MAIT and DN MAIT cells in PBMC of ME/CFS and control subjects. Groups were compared by Mann-Whitney test for non-parametric data, with exact p values, average (AVG) and median (MED) values are shown. (h) The ratio of MAIT cell subset (CD8+ or DN separately) frequency at day 0 to day 6, was correlated with CD27- MAIT cell frequency (of total CD8+ MAIT cells). Data from healthy controls (Healthy, n = 91) and ME/CFS patients (ME/CFS, n = 190) for b, from Healthy (n = 90) and ME/CFS (n = 195) for c (left and middle), from Healthy (n = 90) and ME/CFS (n = 196) for c (right), from Healthy (n = 90) and ME/CFS (n = 186) for d (left), from Healthy (n = 90) and ME/CFS (n = 190) for d (middle), from Healthy (n = 90) and ME/CFS (n = 189) for d (right), from Healthy (n = 91) and ME/CFS (n = 190) for e (left), from Healthy (n = 90) and ME/CFS (n = 196) for e (right), from Healthy (n = 91) and ME/CFS (n = 190) for g, from Healthy (n = 90) and ME/CFS (n = 184) for h (left), from Healthy (n = 60) and ME/CFS (n = 108) for h (right), and groups were compared by Mann-Whitney test for non-parametric data, with exact p-values and average (AVG) and median (MED) values are shown. Correlations of data were performed using nonparametric Spearman correlation, with exact r_s and p value shown.

Figure 7. Activation of MAIT cell effector functions. (a) PBMC were stimulated with combination of the cytokines IL-12+IL-15+IL-18 for 1 day as described in methods, and intracellularly stained for IFNγ and Granzyme A expression, which was analyzed after gating on MAIT (CD161+Vα7.2+) and non-MAIT (CD161-Vα7.2-) CD8+ T cells. (b) Proportion of IFNγ and (c) Granzyme A in CD8+ MAIT and non-MAIT cells from ME/CFS and control subjects. (d) The frequency of CD8+CD45RO+CD27- MAIT cells was correlated to CD8+ MAIT and non-MAIT IFNγ+ cells after stimulation with cytokine combination. Groups were compared by nonparametric Spearman correlation, with exact r_s and p value shown in figures. (e) PBMC were cultured in IL-7 for 6 days (d6) then stimulated with PMA and Ionomycin as described in methods. Frequency of IFNγ and IL-17A expression within MAIT and non-MAIT CD8+ T cells were compared between patient and control groups. (f) IFNγ and TNFα expression, after activation, in CD8+ MAIT, and CD8+CD45RO+ non-MAIT memory T cells. (g) Expression of IFNγ or IL-17+IFNγ cells within CD8+ MAIT cells in ME/CFS and control subjects. (h) Proportion of CD8+ or CD8+ memory (gated on CD45RO+) cells expressing IFNγ. Data from healthy controls (Healthy, n = 91) and ME/CFS patients (ME/CFS, n = 198) for b and c, from Healthy (n = 91) and ME/CFS (n = 185) for d (left), from Healthy (n = 91) and ME/CFS (n = 191) for d (right), from Healthy (n = 91) and ME/CFS (n = 188) for g (left), from Healthy (n = 90) and ME/CFS (n = 183) for g (right), from Healthy (n = 90) and ME/CFS (n = 196) for h, and groups were compared by Mann-Whitney test for non-parametric data, with exact p-values, average (AVG) and median (MED) values are shown. Correlations of data were performed using nonparametric Spearman correlation, with exact r_s and p value shown.

Figure 8. Regulatory T (Treg) cell frequency in ME/CFS. (a) PBMC were stained with Foxp3 and Helios intracellularly and expression was analyzed after gating on CD4+ naïve (CD27+CD45RO-) and memory (CD45RO+) T cells as described in the methods. (b) Proportions of Tregs (Foxp3+Helios+) were calculated within CD4+ naïve and memory subsets in ME/CFS and healthy subjects. (c) CD4+ naïve and memory Tregs divided into two groups based on age younger and older than 50 years in all subjects. (d) Correlation of CD4+ naïve and memory Treg subset frequencies with subject age. (e) Correlation between Th17 cells and memory Tregs was performed by nonparametric Spearman correlation, with exact r_s and p-value shown. The ratio of IL-17-expressing cells within Th17 subset (CCR6+) to memory Tregs were compared between ME/CFS subjects and controls. Data from healthy controls (Healthy, n = 91) and ME/CFS patients (ME/CFS, n = 197) for b, c, and d, from Healthy (n = 80) and ME/CFS (n = 197) for e, and groups compared by the Mann-Whitney test for non-parametric data, with exact p value, average (AVG) and median (MED) values are shown. Correlations of data were performed using nonparametric Spearman correlation, with exact r_s and p value shown.

Figure 9. Random forest clustering of immune features in ME/CFS and control subjects. To generate a receiver operating characteristic (ROC) curve using random forest (RF) clustering algorithm, a training set with 231 samples (80% of total samples) was selected and the remaining data, corresponding to 58 samples (20% of total samples), was left as the test set. Missing values in the training and test sets were replaced by the corresponding median value in the training set. A K-fold cross-validation method was used (K=3) to tube the hyperparameters of the model and was trained using a distinct set of features as input; all 65 immune profile features, the 40 significantly different features, the top 10 significantly different features and the top 10 features that received the highest importance score are plotted.

Tables

Table I: Metrics of the RF classifier using different numbers of immune profile features. The sensitivity (recall), specificity, positive predictive value (precision), negative predictive value, accuracy and F_1 score of immune features are shown. Detailed explanation of these metrics and formulas to calculate them are given in methods section. The rows represent the metrics of: 1) RF classifier when all 65 features are used, 2) for 40 significantly different immune features, 3) the 10 features with the highest importance score among the 40 significantly different immune parameters, and 4) the RF classifier when the top 10 significantly different features are used.

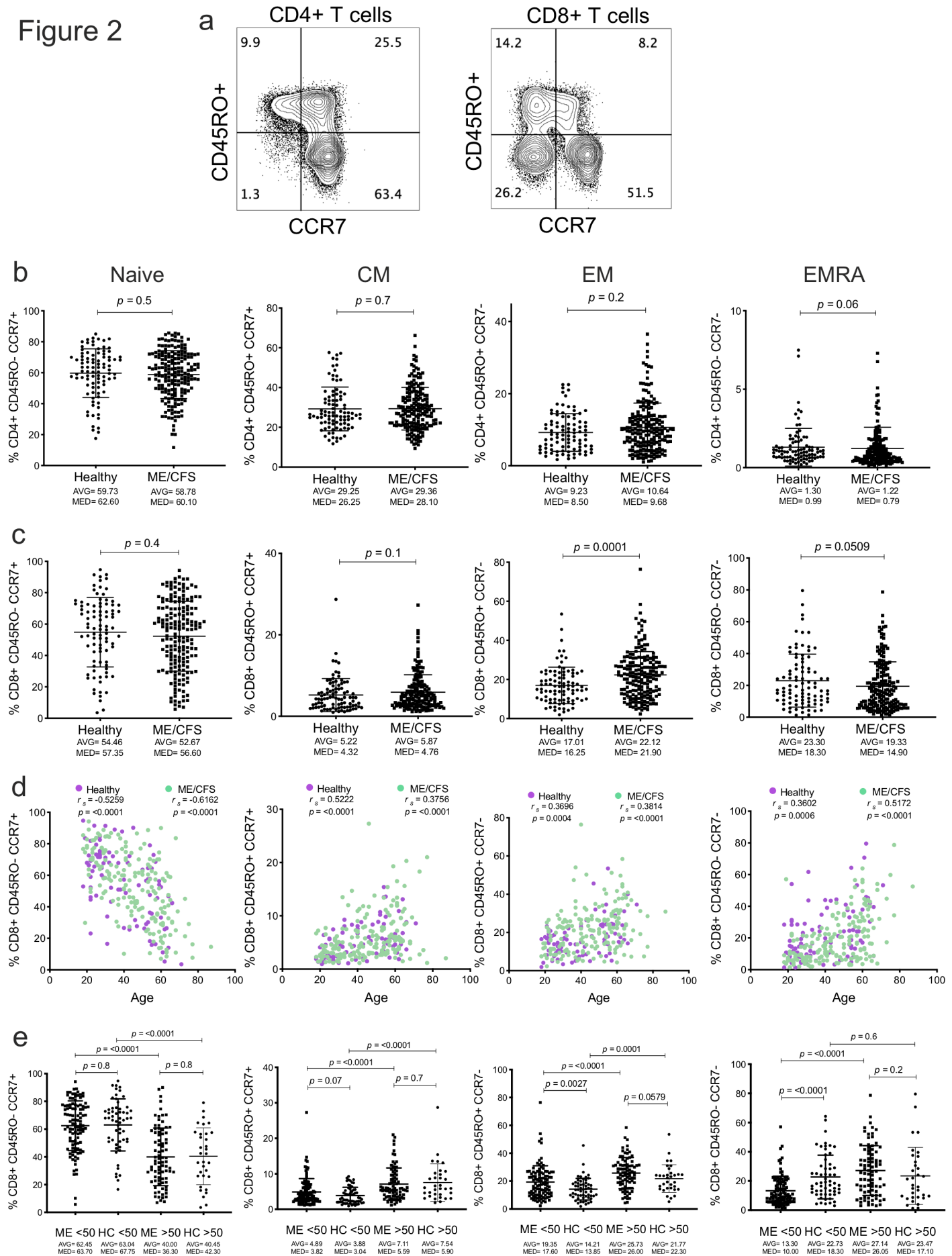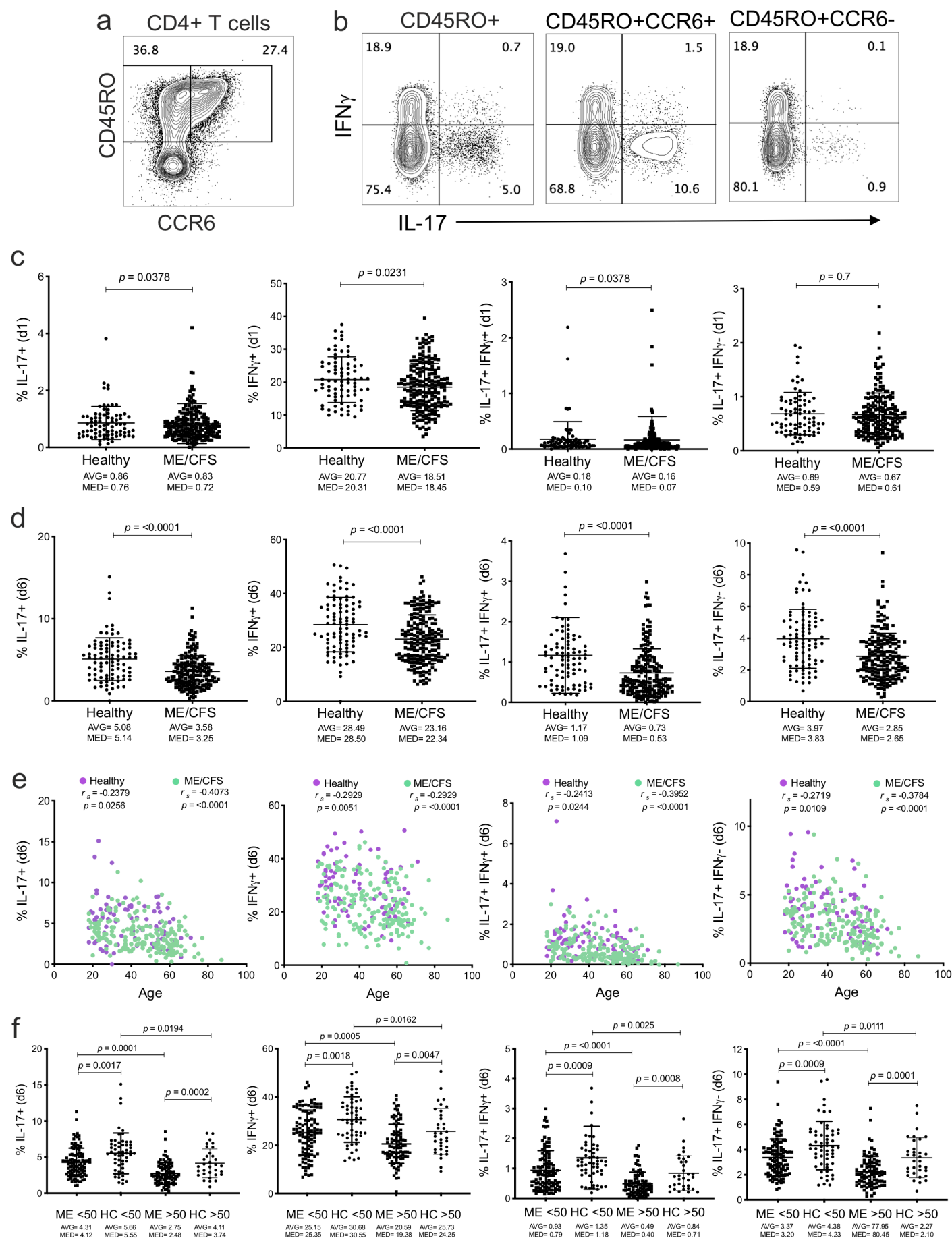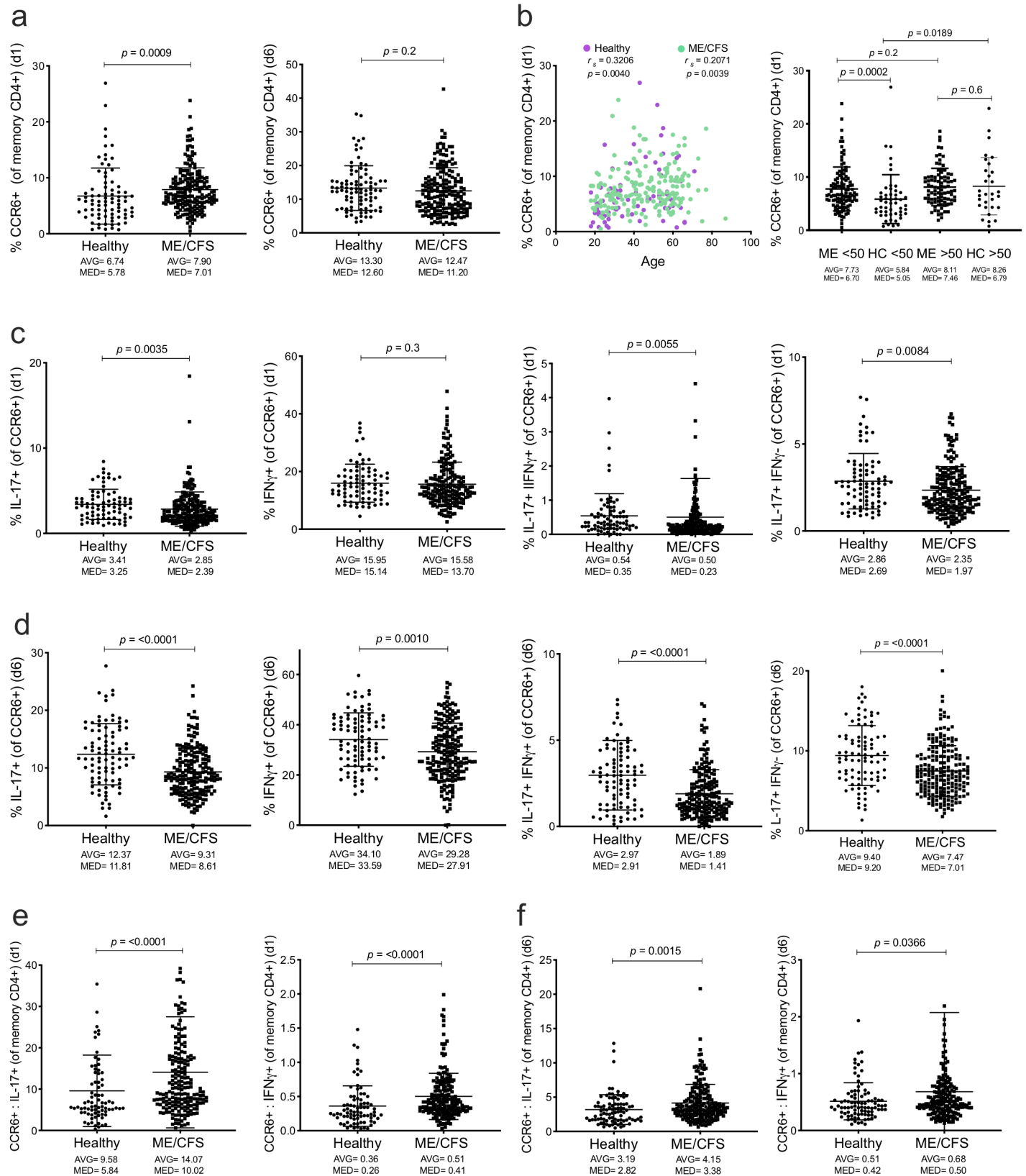| Number of features	Sensitivity	Specificity	Positive pred. value	Negative pred. value	Accuracy	F_1 score	AUC
65 total set	0.950	0.611	0.844	0.846	0.845	0.894	0.929
40 significant	0.900	0.722	0.878	0.765	0.845	0.889	0.915
10 important	0.925	0.556	0.822	0.769	0.810	0.871	0.879
10 significant	0.825	0.722	0.868	0.650	0.793	0.846	0.815

1004
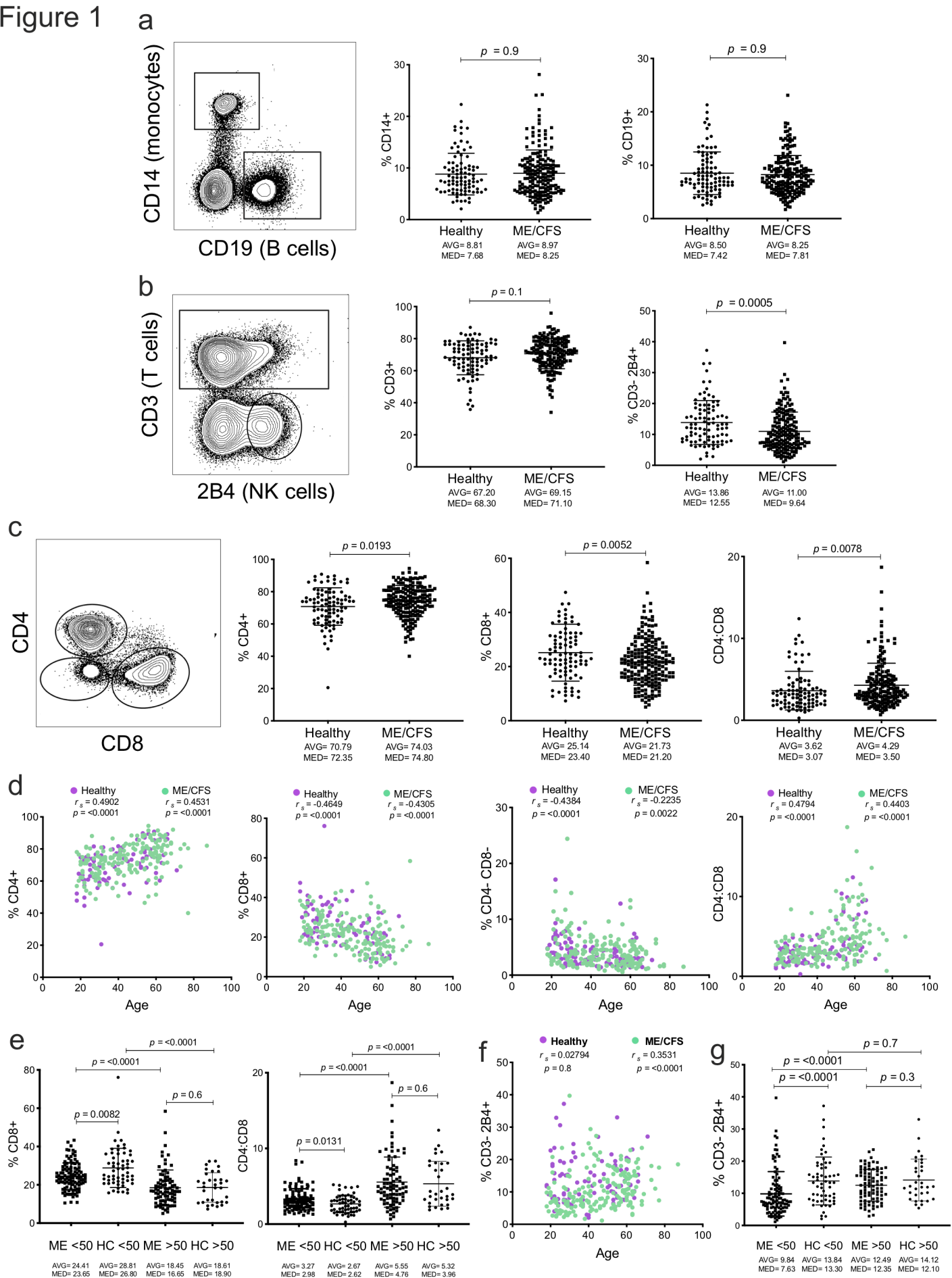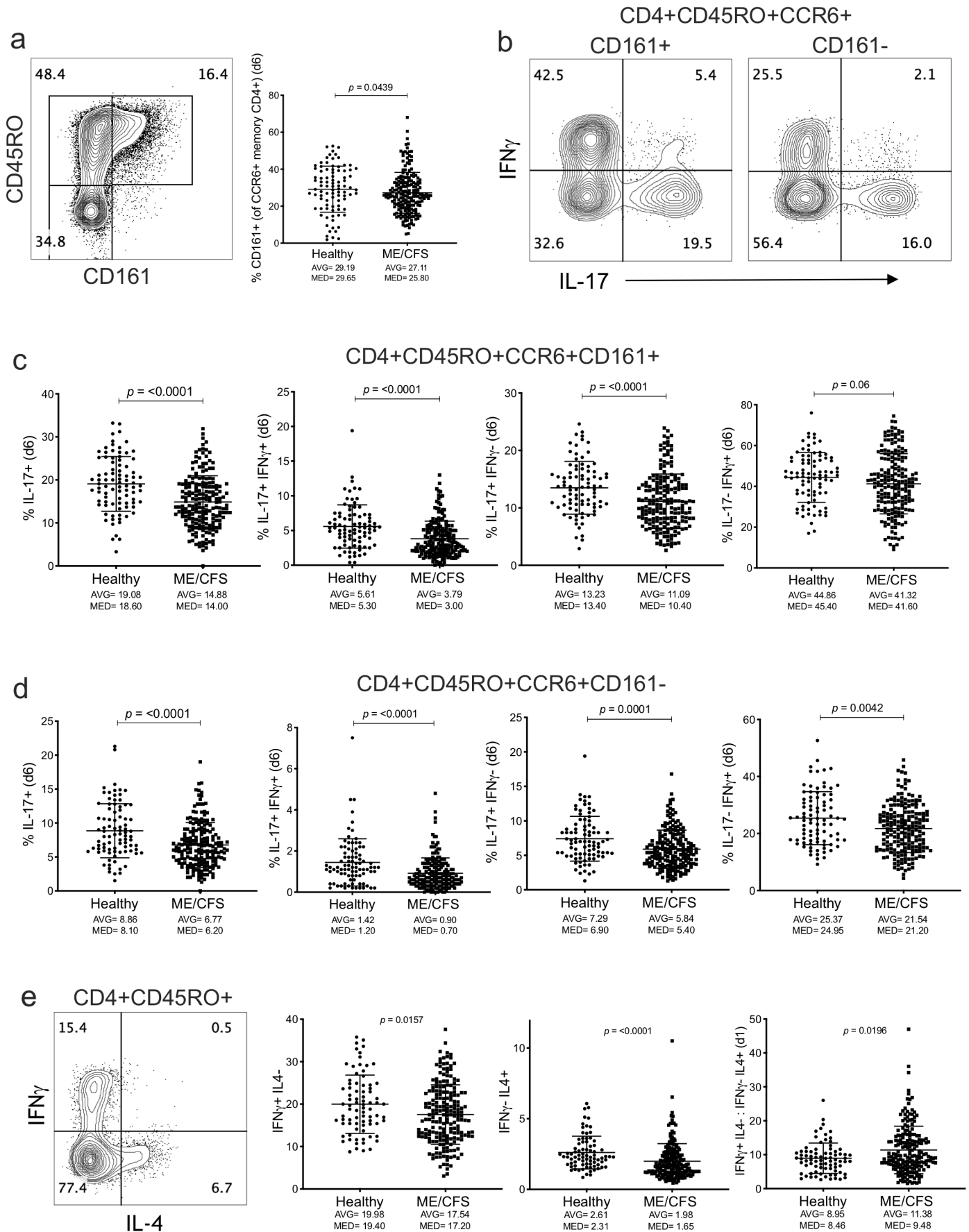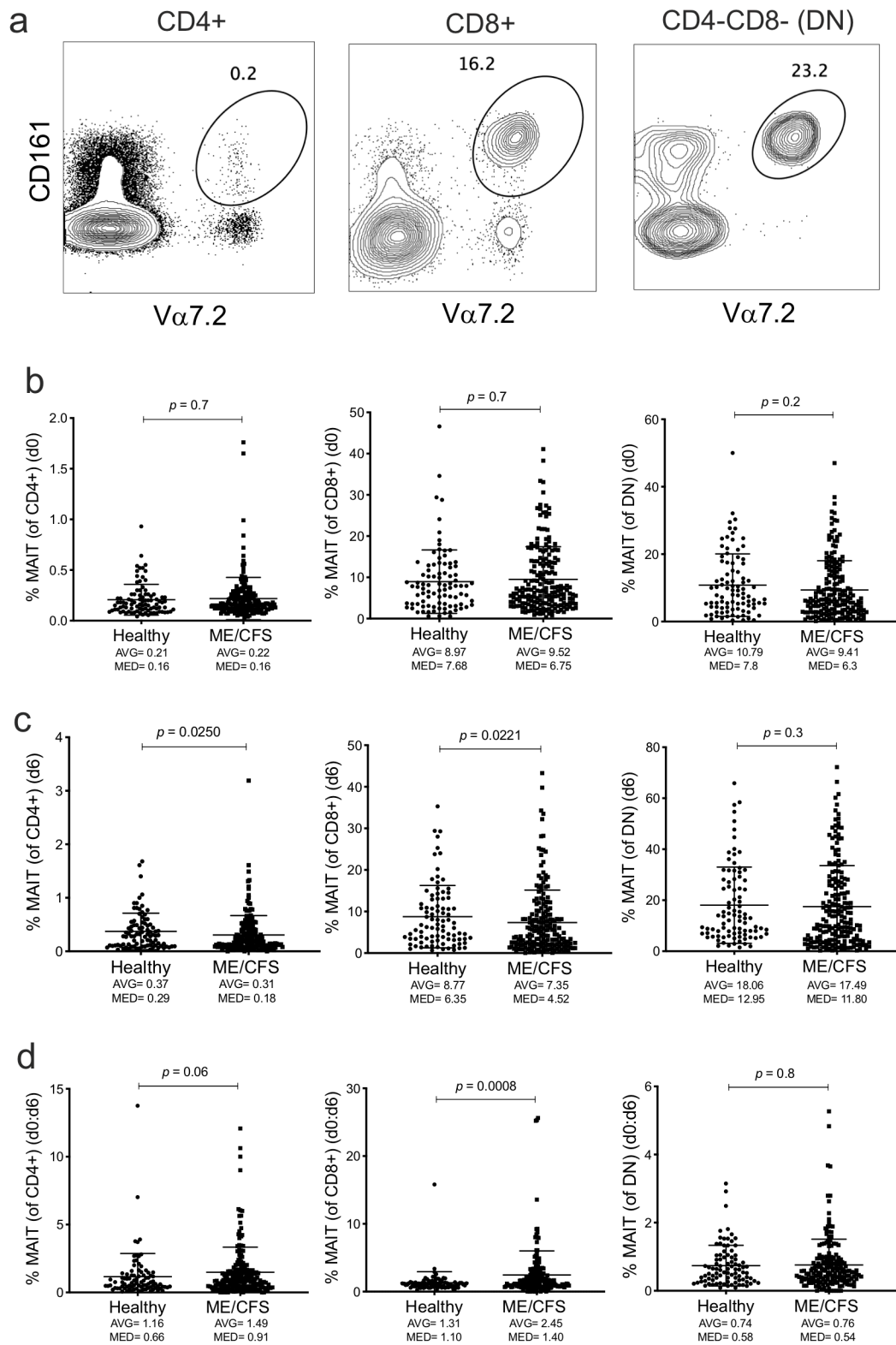
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6 (a-d)

Figure 6 (e-h)

Figure 7 (a-d)

Figure 7 (e-h)

e

CD8+ MAIT

| 81.7 | 11.6 |
| 5.6 | 1.1 |

IFNγ / IL-17A

CD8+ non-MAIT

| 23.8 | 0.2 |
| 75.8 | 0.2 |

f

CD8+ MAIT

| 0.5 | 96.8 |
| 0.3 | 2.4 |

IFNγ / TNF

CD8+CD45RO+

| 5.0 | 77.8 |
| 5.0 | 12.1 |

g

% IFNγ+ (of CD8+ MAIT) (d6)
p = 0.5

Healthy AVG= 91.36 MED= 93.75
ME/CFS AVG= 86.67 MED= 93.78

% IL-17+ IFNγ+ (of CD8+ MAIT) (d6)
p = 0.0075

Healthy AVG= 7.30 MED= 6.37
ME/CFS AVG= 6.08 MED= 5.32

h

% IFNγ+ (of CD8+) (d6)
p = 0.0057

Healthy AVG= 49.74 MED= 51.42
ME/CFS AVG= 42.43 MED= 40.62

% IFNγ+ (of memory CD8+) (d6)
p = 0.0002

Healthy AVG= 74.20 MED= 76.53
ME/CFS AVG= 66.26 MED= 70.00

Figure 8

Figure 9