

Increased expression of activation antigens on CD8+ T lymphocytes in Myalgic Encephalomyelitis/ chronic fatigue syndrome: inverse associations with lowered CD19+ expression and CD4+/CD8+ ratio, but no associations with (auto)immune, leaky gut, oxidative and nitrosative stress biomarkers

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Abstract

BACKGROUND: There is now evidence that specific subgroups of patients with Myalgic Encephalomyelitis / chronic fatigue syndrome (ME/CFS) suffer from a neuro-psychiatric-immune disorder. This study was carried out to delineate the expression of the activation markers CD38 and human leukocyte antigen (HLA) DR on CD4+ and CD8+ peripheral blood lymphocytes in ME/CFS.

METHODS: Proportions and absolute numbers of peripheral lymphocytes expressing CD3+, CD19+, CD4+, CD8+, CD38+ and HLA-DR+ were measured in ME/CFS (n=139), chronic fatigue (CF, n=65) and normal controls (n=40).

RESULTS: The proportions of CD3+, CD8+, CD8+CD38+ and CD8+HLA-DR+ were significantly higher in ME/CFS patients than controls, while CD38+, CD8+CD38+, CD8+HLA-DR+ and CD38+HLA-DR+ were significantly higher in ME/CFS than CF. The percentage of CD19+ cells and the CD4+/CD8+ ratio were significantly lower in ME/CFS and CF than in controls. There were highly significant inverse correlations between the increased expression of CD38+, especially that of CD8+CD38+, and the lowered CD4+/CD8+ ratio and CD19+ expression. There were no significant associations between the flow cytometric results and severity or duration of illness and peripheral blood biomarkers of oxidative and nitrosative stress (O&NS, i.e. IgM responses to O&N modified epitopes), leaky gut (IgM or IgA responses to LPS of gut commensal bacteria), cytokines (interleukin-1, tumor necrosis factor- α), neopterin, lysozyme and autoimmune responses to serotonin.

CONCLUSIONS: The results support that a) increased CD38 and HLA-DR expression on CD8+ T cells are biomarkers of ME/CFS; b) increased CD38 antigen expression may contribute to suppression of the CD4+/CD8+ ratio and CD19+ expression; c) there are different immune subgroups of ME/CFS patients, e.g. increased CD8+ activation marker expression versus inflammation or O&NS processes; and d) viral infections or reactivation may play a role in some ME/CFS patients.

INTRODUCTION

There is evidence that some people with chronic fatigue syndrome (CFS), also labeled Myalgic Encephalomyelitis (ME), suffer from a neuro-psychiatric and physio-somatic disorder characterized by neuro-psychiatric symptoms such as concentration difficulties, failing memory, irritability, sadness, depression, sleep disturbances and autonomic disturbances; and physio-somatic symptoms, including fatigue, a flu-like malaise, headache, irritable bowel and muscle pain (Maes 2013; 2014; Maes *et al.* 2012b; 2013a; Morris & Maes 2013b; Anderson *et al.* 2014). Moreover, ME/CFS is accompanied by (auto)immune-inflammatory alterations, including signs of immune activation and immunosuppression, oxidative and nitrosative stress (O&NS) and specific CNS disorders as measured by different brain imaging techniques (Maes 2013; Maes & Twisk 2010; Morris & Maes 2013b; 2013c).

Psychiatric symptoms in ME/CFS, such as depression, are accompanied by an increased activation of O&NS pathways as measured by IgM responses directed against a number of O&NS-modified neopeptides (Maes *et al.* 2007; 2012a). Immune-inflammatory pathways, including increased levels of inflammatory biomarkers, such as interleukin-1 (IL-1), tumor necrosis factor (TNF)- α , lysozyme and elastase, and markers of cell-mediated immunity, such as neopterin, are significantly correlated with neuropsychiatric (e.g. sadness, autonomic and neurocognitive symptoms), immune (e.g. a flu-like malaise) and physio-somatic (e.g. fatigue) symptoms (Maes *et al.* 2012d; 2013b). Physio-somatic symptoms such as irritable bowel are accompanied by other immune alterations including increased IgA / IgM responses to LPS of gram-negative bacteria, suggesting increased translocation of gram-negative bacteria due to leaky gut (Maes *et al.* 2007; 2012c; 2014; Anderson *et al.* 2014). Increased autoimmune responses to serotonin (5-HT) in ME/CFS are related to neuropsychiatric and inflammatory symptoms (Maes *et al.* 2013b; Anderson *et al.* 2014). We have reviewed elsewhere that there are many different trigger factors which may cause ME/CFS by activating the abovementioned pathways, e.g. viral and bacterial

infections, mold neurotoxins, psychosocial stressors, (auto)immune disorders, inflammatory conditions, etc. (Maes & Twisk 2010; Morris & Maes 2013a; Morris *et al.* 2015).

Immune activation in ME/CFS was already demonstrated in 1991 when Landay *et al.* (1991) reported an increased expression of activation markers, i.e. CD38 and human leukocyte antigen (HLA)-DR, on CD8+ (T suppressor) cells. Both HLA-DR and CD38 are established activation antigens expressed on CD8+ lymphocytes for example during HIV infection and the acute phase of Epstein Barr virus (EBV) and Cytomegalovirus (CMV) infections (Kestens *et al.* 1992; Lynne *et al.* 1998; Doisne *et al.* 2004). CD38 is a multifunctional surface glycoprotein and ectoenzyme (ADP-ribosyl cyclase / cyclic ADP-ribose hydrolase), which is expressed by leukocytes including CD4+ (T helper) and CD8+ cells (Orciani *et al.* 2008; Malavasi *et al.* 2008). CD38 is a receptor signaling molecule that plays a role in cell activation and defense from pathogens (Martins Filho 2011). An altered CD38+ expression is a biomarker for human disorders, such as myelomas and leukemias, viral infections, metabolic disorders and behavioral changes, including autism and chronic fatigue (Landay *et al.* 1991; Malavasi *et al.* 2008; Martins Filho 2011; Higashida *et al.* 2012). For example, increased expression of the CD38 molecule on peripheral blood CD8+ T cells is a biomarker for active hepatitis B infection (Cao *et al.* 2011), CMV infections after kidney transplantation and EBV (Belles-Isles *et al.* 1998; Ticha *et al.* 2010; Lino *et al.* 2011). CD8+CD38+ expression is a biomarker of viral activity and immunological status in HCV+ patients undergoing interferon treatment (Perrella *et al.* 2000). A CD8+CD38+ activated pattern is also observed in HIV infection and is associated with HIV disease progression to AIDS (Liu *et al.* 1997; Holub *et al.* 2004; Romeiro *et al.* 2011). Interestingly, in HIV infection, the elevated expression of CD38 on CD8+ T-lymphocytes is inversely correlated with lowered CD4+ counts, suggesting that chronic activation of CD8+ T-lymphocytes may be responsible at least in part for the loss of CD4+ T cells (Beran *et al.* 2003; Holub *et al.* 2004; Wilson *et al.* 2004; Steel *et al.* 2008).

The aims of this study were to examine a) whether the expression of activation markers, i.e. CD38 and HLA-DR, are elevated on CD8+ and CD4+ T cells in patients with ME/CFS; b) whether there are inverse associations between the increased expression of CD8+CD38+ T cells and lowered levels of CD4+ or CD19+ cells; and c) whether an increased expression of activation markers is associated with (auto)immune (against serotonin), immune-inflammatory (plasma/serum levels of IL-1, TNF- α , lysozyme, neutrophil elastase and neopterin), gut permeability (IgA/IgM response to LPS of commensal bacteria), oxidative stress (IgM responses to oxidatively modified neopeptides) and nitrosative stress (IgM responses to NO-modified epitopes) pathways.

SUBJECTS AND METHODS

Subjects

Two hundred and forty-four subjects participated in this study, i.e. 139 patients with ME/CFS, 65 with chronic fatigue (CF) and 40 controls. We made the diagnosis "ME/CFS" employing the diagnostic criteria of the Centres for Disease Control and Prevention (CDC) (Fukuda *et al.* 1994). Subjects who had suffered from chronic fatigue for more than 6 months but did not fulfil the ME/CFS criteria were diagnosed as suffering from a minor subtype of ME/CFS, denoted as "CF". Patients were admitted to the Maes clinics (Belgium). Controls were recruited by word of mouth from laboratory personnel or their family members. Also subjects who attended our clinics for an O&NS biomarker check-up were included as controls if they fulfilled the in- and exclusion criteria. We excluded the following subjects: a) those with a life-time diagnoses of psychiatric axis-1 disorders (DSM-IV-TR), e.g. psychotic disorders, bipolar disorder, substance dependence/abuse (including tobacco), organic mental disorders; and axis-II diagnoses, i.e. personality disorders, including borderline and schizoid personality disorder; b) those with medical illnesses, including epilepsy, diabetes type I, inflammatory bowel disease, COPD, rheumatoid arthritis; lupus erythematosus, etc.; c) subjects who were treated with anticonvulsants, mood stabilizers, anti-psychotic drugs, antivirals, antibiotics, immunosuppressant drugs (e.g. glucocorticoids) and daily high-dose antioxidant supplements; and d) subjects who had infectious or allergic reactions the last two months prior to inclusion.

Severity of illness was measured with the Fibromyalgia and Chronic Fatigue Syndrome Rating Scale (FF scale) (Zachrisson *et al.* 2002). The FF scale measures 12 key symptoms of ME/CFS, i.e. muscle pain, muscular tension, fatigue, concentration difficulties, failing memory, irritability, sadness, sleep disturbances, autonomic disturbances, irritable bowel, headache, and a flu-like malaise. The total sum on the FF scale was used as a measure of severity of ME/CFS. The study has been approved by the local ethical committee. Subjects gave written informed consent after the study protocol was fully explained.

Methods

Serum and plasma for the assay of white blood cell (WBC) count, flow cytometric leukocyte differentiation, (auto)immune, oxidative and nitrosative stress biomarkers was sampled between 8.30 a.m. and 11.30 a.m. Dual-platform based peripheral blood lymphocyte phenotyping was performed. WBC count was carried out on a fully automated blood cell counter Advia 2120i (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). Flow cytometry of peripheral blood leukocytes was performed using a FACSCalibur (BD Biosci-

ences, Erembodegem, Belgium) flow cytometer using the Cellquest software. Labeled monoclonal antibodies were purchased from BD Biosciences and the following monoclonal antibody sets were used: 1) Multitest CD3 FITC / CD16+56 PE / CD45PerCP / CD19 APC; 2) Multitest CD3 FITC / CD8 PE / CD45 PerCP / CD4 APC; 3) Multitest CD8 FITC / CD38 PE / CD3 PerCP / Anti-HLA-DR APC. In short, 50 µl of EDTA blood was mixed with 15 µl of an antibody set and incubated for 15 minutes at room temperature in the dark. The red blood cells were lysed with a lysing solution (FACS Lysing Solution, BD Biosciences) for 10 min at room temperature in the dark. Afterwards the lymphocytes were analyzed on a FACSCalibur flow cytometer. The lymphocyte subsets were expressed as proportion of positive lymphocytes and absolute number of cells bearing the surface markers.

IgA and IgM-mediated immune responses against the LPS of commensal bacteria (i.e. sum of Z values of the IgM or IgA responses to LPS of *Hafnei Alvei*, *Pseudomonas Aeruginosa*, *Morganella Morganii*, *Pseudomonas Putida*, *Citrobacter Koseri*, and *Klebsiella Pneumoniae*), the serum/plasma levels of IL-1, TNFα, neopterin, elastase and lysozyme, IgG/IgM autoimmune responses directed against serotonin (entered as present or not using a 3 SD cut off value) and IgM responses to oxidatively modified epitopes (i.e. sum of the Z values of IgM responses to malondialdehyde, azelaic acid, phosphatidylinositol and oleic acid) and nitrosative stress (i.e. sum of the Z values of IgM responses to NO-cysteinyl, NO-tryptophan, NO-tyrosine and NO-arginine) were assayed as described previously (Maes & Leunis 2014; Maes *et al.* 2012a; 2012c; 2012d; 2013b).

Statistics

Analyses of variance (ANOVA) were used to check differences in continuous variables between the three diagnostic groups. Analyses of contingency tables (X^2 test) were used to assess the association between categorical variables. Multivariate general linear model (GLM) analyses were used to assess the effects of explanatory variables (e.g. age, sex, diagnosis, biomarkers) on the dependent variables (e.g. the flow cytometric measurements). If the multivariate tests were significant, we used tests of between-subject effects to analyze the effects of the significant predictor variables on the dependent variables. Main post-hoc differences between the subgroups were checked using Bonferroni's adjusted comparisons. Automatic multinomial logistic regression analysis was used to delineate the significant predictor variables (i.e. flow cytometric results) that are associated with ME/CFS and CF (dependent variables) with controls as reference group. We employed the IBM SPSS (Windows version 22) to analyze the data. Statistical significance was set at $p=0.05$, two tailed.

RESULTS

Table 1 shows the mean values of the demographic, clinical and flow cytometric data. There were no significant differences in age ($F=0.90$, $df=2/241$, $p=0.407$) or gender ($X^2=4.38$, $df=2$, $p=0.112$) between the three study groups. There was no significant difference in duration of illness between patients with CF and ME/CFS ($F=0.62$, $df=1/139$, $p=0.434$). The total FF score was significantly higher in subjects with ME/CFS than in those with CF ($F=78.8$, $df=1/195$, $p<0.001$). The numbers of leukocytes ($F=0.81$, $df=2/241$, $p=0.447$) and lymphocytes ($F=1.9$, $df=12/241$, $p=0.149$) were not significantly different between the three subgroups. We do not show the results of multiple statistical analyses on the flow cytometric data as these were evaluated only when the multivariate GLM analyses were significant. If significant, the multivariate GLM analysis was followed by tests of between-subject effects.

Table 2 shows the results of a multivariate GLM analysis with the 12 flow cytometric measurements as dependent variables and diagnosis (i.e. entered as three groups that is controls, CF and ME/CFS), gender, the interaction diagnosis X gender, and age as explanatory variables. The GLM analyses showed significant multivariate effects of diagnosis, age and gender. Tests of between-subject effects showed significant

Tab. 1. Demographic and clinical data and results of white blood cell counts and flow cytometric results in patients with chronic fatigue (CF), Myalgic Encephalomyelitis / chronic fatigue syndrome (ME/ CFS) and normal controls (NC).

Variables	NC	CF	ME/CFS
Age (years)	42.6 (10.6)	41.6 (13.2)	39.9 (12.5)
Gender (female/male)	27 / 13	51 / 14	115 / 24
total FF score	-	25.4 (9.6)	39.0 (10.2)
Duration of illness (years)	-	4.2 (6.6)	5.0 (5.0)
Leukocytes μL	6873 (1576)	6674 (1845)	7022 (1892)
Lymphocytes μL	2160 (516)	1950 (511)	2045 (551)
CD3+ %	70.8 (12.0)	72.5 (5.9)	74.2 (6.3)
CD4+ %	50.0 (9.4)	48.0 (8.1)	47.9 (7.2)
CD8+ %	21.1 (6.7)	22.7 (7.1)	24.9 (7.8)
CD4+/CD+ ratio	2.88 (2.40)	2.39 (1.03)	2.17 (0.92)
CD19+ %	14.2 (6.2)	13.2 (4.5)	13.2 (4.4)
CD38+ %	31.7 (9.7)	30.7 (7.8)	35.7 (8.6)
CD4+CD38+ %	26.5 (8.5)	24.5 (6.4)	27.2 (7.0)
CD8+CD38+ %	5.7 (2.4)	6.4 (3.0)	8.8 (5.0)
HLA-DR+ %	5.1 (2.2)	5.2 (2.6)	6.4 (5.3)
CD4+HLA-DR+ %	3.3 (1.6)	3.3 (1.5)	3.6 (3.4)
CD8+HLA-DR+ %	1.9 (0.9)	2.2 (1.8)	3.0 (3.1)
CD38+HLA-DR+ %	2.3 (1.0)	2.0 (1.3)	3.0 (3.3)

effects of diagnosis on CD3+, CD8+, CD19+, CD38+, CD8+CD38+, CD8+HLA-DR+ and CD38+HLA-DR+ subset proportions and the CD4+/CD8+ ratio. The proportions of CD3+, CD8+, CD8+CD38+ and CD8+HLA-DR+ were significantly higher in ME/CFS patients than in controls, while the CD38+, CD8+CD38+, CD8+HLA-DR+ and CD38+HLA-DR+ were significantly higher in ME/CFS than in CF. The percentage of CD19+ cells and the CD4+/CD8+ ratio were significantly lower in ME/CFS and CF than in controls. Tests of between-subjects effects showed significantly higher CD3+, CD4+, CD38+ and CD4+CD38+ cell numbers in women as compared to men. There were significantly positive associations between age and CD4+ numbers and the CD4+/CD8+ ratio, and significant negative associations between age and CD8+, CD38+ and CD8+CD38+ numbers.

We re-ran the same multivariate GLM analysis with the number of lymphocytes as an additional explanatory variable. The results show that the multivariate effects of lymphocyte number ($F=2.68$, $df=12/163$, $p=0.003$), diagnosis ($F=1.65$, $df=24/328$, $p=0.031$), age ($F=6.61$, $df=12/163$, $p<0.001$) and gender ($F=2.32$, $df=12/163$, $p=0.009$) were significant. The tests of between-subjects effects showed similar results as those presented in Table 2.

Table 3 shows the results of an automatic stepwise multinomial logistic regression analysis with ME/CFS and CF as dependent variables (and controls as reference group) and all 12 flow cytometric measurements as explanatory variables. There were two significant explanatory variables ($X^2=27.20$, $df=4$, $p<0.001$, Nagelkerke=0.165), i.e. CD3+ ($X^2=7.24$, $df=1$, $p=0.027$) and CD8+CD38+ ($X^2=12.90$, $df=1$, $p=0.002$). Table 3 shows the parameter estimates. CD8+CD38+ was the only variable that also separated ME/CFS from CF ($X^2=8.00$, $df=1$, $p=0.005$).

Table 4 shows the results of multivariate GLM analysis with the 12 flow cytometric measurements as dependent variables and gender and age and the listed variables as explanatory variables. We found that only elastase was significantly associated with the flow cytometric measurements. Tests of between-subject effects showed that elastase was significantly and positively correlated only with the CD4+HLA-DR+ proportion ($F=12.45$, $df=1/82$, $p=0.001$).

Table 5 shows the results of multivariate GLM analyses with the CD4+ and CD19+ cell proportions and the CD4+/CD8+ ratio as dependent variables and the CD38+ and CD8+CD38+ proportions as explanatory variables. The multivariate test showed a significant effect of CD38+ and CD8+CD38+ on the dependent variables. Tests of between-subject effects showed that CD4+ proportion and the CD4+/CD8+ ratio were inversely related to CD8+ and CD8+CD38+ and that the CD19+ proportion was inversely related to CD8+CD38+. We have also examined the associations between the absolute numbers of CD4+ and CD19+

Tab. 2. Results of multivariate GLM analysis with the 12 proportions of lymphocyte subsets as measured by flow cytometry as dependent variables and diagnosis (entered as three groups), gender and age as explanatory variables.

Tests	Dependent variables	Explanatory variables	F	df	p-value
Multivariate tests	All 12 flow cytometric data (proportions)	Diagnosis	1.64	24 / 330	0.031
		Gender	2.35	12 / 164	<0.001
		Age	6.38	12 / 164	<0.001
		Diagnosis X gender	1.26	24 / 330	0.190
Tests of between-subject effects	CD3+ %	Diagnosis (ME/CFS > NC)*	5.54	2 / 175	0.005
	CD8+ %	Diagnosis (ME/CFS > NC)*	4.03	2 / 175	0.020
	CD4+/CD8+ ratio	Diagnosis (ME/CFS and CF < NC)*	5.05	2 / 175	0.007
	CD19+ %	Diagnosis (ME/CFS and CF < NC)*	4.19	2 / 175	0.017
	CD38+ %	Diagnosis (ME/CFS > CF)*	4.02	2 / 175	0.020
	CD8+CD38+ %	Diagnosis (ME/CFS > NC and CF)*	8.68	2 / 175	<0.001
	CD8+HLA-DR+ %	Diagnosis (ME/CFS > NC and CF)*	4.61	2 / 175	0.011
Tests of between-subject effects	CD3+ %	Gender (F > M)	14.08	1 / 175	<0.001
	CD4+ %	Gender (F > M)	4.10	1 / 175	0.044
	CD38+ %	Gender (F > M)	9.81	1 / 175	0.002
	CD4+CD38+ %	Gender (F > M)	11.00	1 / 175	0.001
Tests of between-subject effects	CD4+ %	Age (+)	5.15	1 / 175	0.024
	CD8+ %	Age (-)	11.86	1 / 175	0.001
	CD4+/CD8+ ratio	Age (+)	10.56	1 / 175	0.001
	CD38+ %	Age (-)	7.52	1 / 175	0.007
	CD8+CD38+ %	Age (-)	30.84	1 / 175	<0.001

Diagnosis: ME/CFS: Myalgic Encephalomyelitis / chronic fatigue syndrome, CF: chronic fatigue, and NC: normal controls; F: female, M: male. *Results of Bonferroni corrected post-hoc tests. Age (+) and (-): denotes the sign of the correlation

Tab. 3. Results of multinomial logistic regression analysis with Myalgic Encephalomyelitis / chronic fatigue syndrome (ME/CFS) and chronic fatigue (CF) as dependent variables (normal controls - NC as reference group) and the flow cytometric measurements (proportions) as explanatory variables.

Contrast	Significant explanatory variables	B	SE	Wald	df	p-value	Odds ratio	95% CI lower - upper
ME/CFS versus NC	CD8+CD38+ %	0.209	0.093	5.02	1	0.025	1.23	1.03 - 1.48
	CD3+ %	0.081	0.034	5.53	1	0.019	1.08	1.01 - 1.16
CF versus NC	CD8+CD38+ %	0.051	0.097	0.28	1	0.599	1.05	0.87 - 1.27
	CD3+ %	0.053	0.032	2.69	1	0.101	1.06	0.99 - 1.12

CI: confidence intervals.

cells and the CD4+/CD8+ ratio as dependent variables and the CD8+ and CD8+CD38+ proportions as explanatory variables. The results were basically similar to those reported in Table 5.

DISCUSSION

The first major finding of this study is that the activation markers CD38 and HLA-DR were significantly more expressed on CD8+, but not CD4+, T cells of patients with ME/CFS than normal controls and subjects with CF. Especially the expression of CD38 was highly increased on CD8+ T cells of patients with ME/CFS. Our findings in part replicate the findings by Landay *et al.* (1991) who reported increased CD8+CD38+ and CD8+HLA-DR+ cell proportions in ME/CFS patients. However, these authors also found reduced CD8+ cells, whereas we found increased CD8+ T cell proportions in subjects with ME/CFS as compared to controls. Our

findings show that CD8+, but not CD4+, cells are activated in ME/CFS, but not CF, findings which support our view that both groups are immunologically different (Maes *et al.* 2012b). Nevertheless, no significant associations could be found between the increased expression of CD38 and HLA-DR on CD8+ T cells and activation of other immune-inflammatory and O&NS pathways. This could suggest that there are different subgroups of ME/CFS patients which are characterized by a different immune profile, e.g. CD8 activation versus activated immune-inflammatory and O&NS pathways.

We found that ME/CFS and CF are accompanied by lowered levels of CD19+ B cells and a lowered CD4+/CD8+ T cell ratio as compared to normal controls. Thus while some immune functions may be upregulated (e.g. CD8+ T cell activation), other functions are downregulated in ME/CFS (CD19+ expression and CD4+/CD8+ ratio). Also these findings are in accor-

Tab. 4. Outcome of different multivariate GLM analyses with the 12 proportions of lymphocyte subsets as measured by flow cytometry as dependent variables and gender and age as explanatory variables (see Table 2) and each of the listed variables as additional explanatory variable.

Additional explanatory variable	F	df	p-value
Total FF score	1.40	12 / 142	0.174
Duration of illness	0.64	12 / 89	0.805
Interleukin-1	0.85	12 / 78	0.595
Tumor necrosis factor- α	1.00	12 / 88	0.454
Neopterin	1.57	12 / 105	0.111
Elastase	2.61	12 / 71	0.006
IgM responses to LPS of 6 gram-negative bacteria	0.77	12 / 126	0.684
IgA responses to LPS of 6 gram-negative bacteria	1.39	12 / 125	0.177
Autoimmune responses directed against serotonin	1.57	12 / 128	0.109
IgM against oxidatively modified neopeptides	0.70	12 / 125	0.751
IgM against nitrosatively modified neopeptides	0.75	12 / 125	0.696

dance with previous reports that ME/CFS is accompanied by simultaneous signs of immune activation and immunosuppression (Maes & Twisk 2010; Morris & Maes 2013a; 2013c). It should be underscored however that the decreases in CD19+ cells and the CD4+/CD8+ T cell ratio are not clinically relevant.

It is tempting to speculate that the increased expression of CD38 and HLA-DR on CD8+ T cells is associated with putative viral infections or a reactivation, such as with CMV or EBV, which are known to play a role in ME/CFS (Morris *et al.* 2015). As described in the Introduction, the CD38 molecule plays a role in defense from pathogens (Martins Filho 2011), while an increased expression of the CD38 molecule on peripheral blood CD8+ T cells is a biomarker for CMV and EBV infections (Belles-Isles *et al.* 1998; Ticha *et al.* 2010). Recently, we have reviewed the possible role of

CMV infection in the pathogenesis of ME/CFS (Morris *et al.* 2015). CMV infection often causes a syndrome with fatigue, malaise and myalgia and increased production of pro-inflammatory cytokines via an increased transcription of nuclear factor (NF)- κ B and systemic oxidative stress, which are both mechanism driving the development of ME/CFS pathology. Interestingly, CMV may lead to persistent life-long infections accompanied by lowered mitogen-induced T cell responses and natural killer cell activity, other findings in patients with ME/CFS (Morris *et al.* 2015). Also EBV infection may be accompanied by increased CD38 and HLA-DR expression (see Introduction) and all symptoms of infectious ME/CFS with fatigue, intermittent fever, lymph node tenderness, sore throat, myalgia and headache (Morris *et al.* 2015). Future research should examine the expression of CD38 and HLA-DR on CD8+ T cells in ME/CFS patients in relation to viral infections including CMV and EBV. However, it is also possible that the increased expression of the CD8+CD38+ T cells is associated with increased psychological stress in those patients. Indeed, acute psychological stress is accompanied by an acute increase in the expression of CD8+CD38+ cells (Atanackovic *et al.* 2002) and with increased HLA-DR expression but a significantly reduced CD4+/CD8+ T cell ratio (Maes *et al.* 1999).

The second major finding of this study is that increased proportions of CD38+ and CD8+CD38+ are inversely associated with decreased CD4+ and CD19+ proportions or number of cells and with a decreased CD4+/CD8+ ratio in general and with lower levels of CD19+ cells and CD4+/CD8+ ratio in ME/CFS more specifically. As described in the introduction, in HIV+ subjects, the increased expression of CD38 on CD8+ cells is inversely associated with lowered CD4+ numbers (Beran *et al.* 2003; Holub *et al.* 2004; Wilson *et al.* 2004; Steel *et al.* 2008). This could suggest that activation of CD8+ cells is responsible for the marginally lowered levels of CD19+ cells and the CD4+/CD8+ T cell ratio in ME/CFS. In this respect, it is interesting to note that the CD8+CD38+ subpopulations may produce nonlytic suppressive factors with antiviral activity (Jiang *et al.* 2003). In addition, the CD38 molecule demarcates regulatory CD8+ T cells with suppressor

Tab. 5. Results of multivariate GLM analysis with the CD4+ % and CD19+ % proportions and the CD4+/CD8+ ratio as dependent variables and the CD38+ % and CD8+CD38+ % proportions as explanatory variables.

Tests	Dependent variables	Explanatory variables	F	df	p-value
Multivariate tests	CD4+ %, CD19+ % and CD4+/CD8+ ratio	CD38+ %	46.05	3 / 181	<0.001
		CD8+CD38+ %	73.65	3 / 181	<0.001
Tests of between-subject effects	CD4+ %	CD38+ %	134.83	1 / 183	< 0.001
		CD8+CD38+ %	168.42	1 / 183	< 0.001
Tests of between-subject effects	CD4+/CD8+ ratio	CD38+ %	26.48	1 / 183	<0.001
		CD8+CD38+ %	82.72	1 / 183	<0.001
Tests of between-subject effects	CD19+ %	CD38+ %	2.40	1 / 183	0.123
		CD8+CD38+ %	8.63	1 / 183	0.004

activities of excessive immune responses (Bahri *et al.* 2012).

It is interesting to note that age and gender significantly modified our flow cytometry results. Thus, females showed higher proportions of CD3+, CD4+, CD38+ and CD4+CD38+ T cells than men. Age was significantly and positively related to the CD4+ proportion and the CD4+/CD8+ T cell ratio, and negatively to CD8+, CD38+ and CD8+CD38+ T cell proportions. These findings extend the results of Jiao *et al.* (2009) who found that CD8+CD38+ T cells were inversely related to age. However, in contrast to these authors we were unable to find significant inverse relationships between age and the CD3+, CD19+ and CD8+HLA-DR+ levels. These results show that all results of flow cytometry should always be controlled for possible effects of age and sex.

Conflict of interest:

The author does not report any conflict of interest.

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