Cytokine expression provides clues to the pathophysiology of Gulf War illness and myalgic encephalomyelitis

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ABSTRACT

Gulf War illness (GWI) is a chronic disease of unknown etiology characterized by persistent symptoms such as cognitive impairment, unexplained fatigue, pervasive pain, headaches, and gastrointestinal abnormalities. Current reports suggest that as many as 200,000 veterans who served in the 1990–1991 Persian Gulf War were afflicted. Several potential triggers of GWI have been proposed including chemical exposure, toxins, vaccines, and unknown infectious agents. However, a definitive cause of GWI has not been identified and a specific biological marker that can consistently delineate the disease has not been defined. Myalgic encephalomyelitis (ME) is a disease with similar and overlapping symptomology, and subjects diagnosed with GWI typically fit the diagnostic criteria for ME. For these reasons, GWI is often considered a subgroup of ME. To explore this possibility and identify immune parameters that may help to understand GWI pathophysiology, we measured 77 serum cytokines in subjects with GWI and compared these data to that of subjects with ME as well as healthy controls. Our analysis identified a group of cytokines that identified ME and GWI cases with sensitivities of 92.5% and 64.9%, respectively. The five most significant cytokines in decreasing order of importance were IL-7, IL-4, TNF-α, IL-13, and IL-17F. When delineating GWI and ME cases from healthy controls, the observed specificity was only 33.3%, suggesting that with respect to cytokine expression, GWI cases resemble control subjects to a greater extent than ME cases across a number of parameters. These results imply that serum cytokines are representative of ME pathology to a greater extent than GWI and further suggest that the two diseases have distinct immune profiles despite their overlapping symptomology.

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1. Introduction

Gulf War illness (GWI) and myalgic encephalomyelitis (ME) are complex diseases of unknown etiology. They are often characterized by a constellation of unexplained and overlapping symptoms, which include widespread inflammation, fatigue, multisystemic neuropathology, joint and muscle pain and gastrointestinal pathology [1–3]. Although the two diseases are similar with overlapping symptoms, GWI is a specific term given to returning military veterans and civilian workers of the Persian Gulf War that took place from August 2, 1990 to February 28, 1991. ME is frequently associated with acute flu-like onset as well as noninfectious environmental triggers [4]; whereas, multiple factors including environmental exposure, toxins, vaccines, and unknown infectious agents have been evaluated as potential triggers for GWI [5,6]. Indeed, GWI and ME have many clinical symptoms in common including long-term and severe fatigue that is not relieved by rest, gastrointestinal disorders, and neurological impairments [2]. Accordingly, it has been suggested that GWI cases meet the diagnostic criteria for ME and, therefore, represent discrete subsets of ME. Currently, there is no pathognomonic marker for either disease as well as no clinical diagnostic test available; for these reasons, diagnosis is mainly based on clinical observation, epidemiological evaluation, and medical anamnesis.

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Immunological impairments in subjects with ME have been extensively documented. For example, several researchers have reported abnormalities in natural killer (NK) cell numbers and function [7,8] as well as abnormalities in serum and plasma cytokine and chemokine levels [9–12]. Natelson et al. observed that levels of granulocyte–macrophage colony-stimulating factor (GM-CSF) in the cerebral spinal fluid of ME cases were lower than in controls and that levels of CXCL8 were elevated in cases with sudden, influenza-like onset when compared to cases with gradual onset or healthy controls [13]. In a study by Zhang and colleagues, two groups of cases who met the case definition for ME were compared to each other; Gulf War veterans who developed their malady after they had returned home from the Gulf and a group of nonveterans who developed the illness sporadically [14]. They reported that Gulf War veterans with ME had a statistically significant increase in total T cells and a lower percentage of NK cells when compared to respective controls. In addition, veterans with ME had higher levels of interleukin (IL)-2, IL-10, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α over that of controls. However, they observed no difference in civilian veterans with ME when compared to controls across a number of immune parameters.

Unquestionably, serum or plasma inflammatory cytokine and chemokine levels are some of the most commonly reported differences between subjects with ME and healthy controls. For example, Maes et al. reported that subjects with ME have significantly higher levels of serum IL-1 and TNF-α when compared to controls [15,16]. Also, Fletcher and coworkers observed increased levels of serum regulatory and proinflammatory cytokines such as lymphotoxin-α, IL-1α, IL-1β, IL-4, IL-5, IL-6, and IL-12 in ME cases when compared to controls [17]. Other studies have investigated cytokine profiles of subjects with GWI and ME [18]. A Th2 shift is commonly reported for subgroups of ME cases [11,19], suggesting that those with ME may be hyper-responsive to allergens, toxins, extracellular bacteria, and parasites and hypo-responsive to viruses and intracellular bacteria. Smylie et al. reported a decreased Th2 polarity in females with ME as compared to GWI and suggested that an IL-23/Th17/IL-17 axis could be used to delineate GWI and ME [18]. Skowera and colleagues reported that, in contrast to asymptomatic Gulf War veterans, symptomatic veterans with "multisymptom illness" displayed an ongoing Th1-type immune activation with significantly elevated levels of IFN-γ and IL-2, in the absence of in vitro stimulation [20]. These studies suggest that although similar in clinical manifestations, GWI and ME potentially present discrete cytokine profiles, which may reflect differences in disease pathogenesis.

Cytokines orchestrate numerous immune functions including activating and prolonging leukocyte proliferation, directing migration, and influencing and shaping leukocyte functional activity. Abnormal leukocyte counts in subjects with GWI and ME may, in fact, be a consequence of dysregulated cytokine control. Subsequently, abnormal leukocyte counts may lead to a disturbed immune response, often manifesting with broad clinical presentations. It may also be suggested that cytokine profiles, which are reflective of the profound immune disturbances in subjects with GWI and ME, might potentially serve as useful biomarkers. A greater understanding regarding cytokine dysregulation in GWI and ME may also help to better understand the pathogenesis of these diseases, thus improving diagnosis, treatment efficacy, and prophylactic measures.

In the present study, we have conducted a comprehensive survey of 77 different cytokines and chemokines in an effort to better understand the immune responses associated with GWI and ME. Our results suggest that Th1 and Th17 cytokines underscore GWI cases, while Th1 and Th2 cytokines as well as a more diverse group of inflammatory cytokines and mononuclear chemoattractant cytokines characterize ME. Additionally, in order to identify the most important cytokines that distinguish these groups and potentially identify underlying pathology, we utilized the machine logic nearest neighbor predictor algorithm Random Forest to analyze these data. The five most significant cytokines identified by our model in decreasing order of importance were IL-7, IL-4, TNF-α, IL-13, and IL-17F. Although our Random Forest analysis produced a cytokine signature that identified ME cases with 92.5% sensitivity, only 64.9% sensitivity was achieved when delineating GWI cases. Furthermore, specificity was only 33.3%, suggesting that with respect to cytokine expression, GWI cases resemble control subjects to a greater extent than ME cases across a number of parameters. These results imply that serum cytokines are representative of ME pathology to a greater extent than GWI and further suggest that the two diseases have distinct immune profiles despite their overlapping symptomology.

2. Materials and methods

2.1. Study subjects

A total of 146 subjects were enrolled in these studies; 67 cases with a confirmed diagnosis of ME, 37 identified as having GWI, and 42 healthy controls. Informed consent was obtained from each participant according to human subjects protocols approved by the University of Nevada Biomedical Institutional Review Board (protocols B12-031 and B12-036). The cases identified as having ME were physician diagnosed and met the Carruthers et al. criteria for ME as well as the 1994 Fukuda et al. criteria [1,21,22]. ME subjects were recruited from across the United States and from individuals who sought treatment for ME at the Himmunitas ME/CFS clinic in Brussels Belgium. GWI subjects were recruited by the VA Sierra Nevada Health Care System Medical Center in Reno, Nevada, and were physician diagnosed satisfying the inclusion criteria of having been on active duty in the military during the Persian Gulf War (Operation Desert Storm: 1990–1991) and symptoms consistent with GWI as defined by the Centers for Disease Control and Prevention (CDC) and Kansas criteria for GWI [23,24]. Cases were generally representative of the respective populations for each disease based on gender and age.

2.2. Serum samples

Our initial evaluation regarding the method of blood collection indicated that most anticoagulants we tested activated cytokine expression to some level over a 24-h time period (data not shown). The activation was the most pronounced with blood collected on heparin. Given that lymphocytes from ME subjects respond to a greater extent upon stimulation than controls (unpublished observation), this problem would not be normalized even when cases and controls are handled in an identical manner. Additionally, our study required some blood to be shipped overnight; therefore, we chose to conduct our analysis on serum rather than plasma. Whole blood was collected using serum-separator tubes, centrifuged immediately to isolate the serum, and aliquots were made at approximately 24 h post draw and stored at −80 °C until analyzed.

2.3. Cytokine analysis

Serum cytokine levels were analyzed on a Luminex 200 analyzer (Austin, TX) with Bio-Plex (Bio-Rad, Hercules, CA) multiplex magnetic bead-based antibody detection kits according to the manufacturer's instructions. Bio-Plex Pro Human Chemokine panels (40-Plex), Bio-Plex Pro Human Th17 Cytokine panels, Bio-Plex Pro Human Cytokine 27-plex panels, and Bio-Plex Human Cytokine

21-plex panels were used to cover a total of 77 cytokines and chemokines (herein referred to as "cytokines"). For each subject, 50 µl of serum was analyzed and a minimum of 50 beads per cytokine was acquired. Data collected was analyzed using MasterPlex CT control software and MasterPlex QT analysis software (MiraiBio division of Hitachi Software, San Francisco, CA). Standard curves for each cytokine were generated using standards provided by the manufacturer and some samples were analyzed on multiple runs for quality control purposes and to normalize the collective runs.

2.4. Statistical analysis

In order to determine differences in cytokine values and distributions between GWI, ME, and control subjects, we initially performed Kolmogorov–Smirnov tests for normality, which revealed that the data were not normally distributed (data not shown). We therefore used the non-parametric Kruskal–Wallis (K.W.) one-way analysis of variance by ranks to confirm that the three populations did not originate from the same distribution. We then performed a Mann–Whittney (M.W.) analysis to identify differences in medians between GWI and ME cases as well as between GWI cases and controls and ME cases and controls. We additionally conducted Pearson correlation analysis, comparing cytokines to each other within each respective subject population. Finally, we performed classification analysis using the tree-based ensemble machine learning algorithm Random Forest [25]. For this analysis, 500 random trees were built using six predictors for each node, and auto-bootstrap out-of-bag sampling was used for testing the model.

3. Results

3.1. Differential expression of serum cytokines

In these studies, a total of 104 cases (67 ME and 37 GWI) and 42 controls were analyzed for 77 serum cytokines. Subjects classified as having ME were physician diagnosed and fulfilled the criteria

described by Carruthers et al. [1,21] as well as the Fukuda criteria [22]. GWI subjects were physician diagnosed at the VA Sierra Nevada Health Care System Medical Center in Reno, Nevada, and met the inclusion criteria as having been on active duty in the military during the Persian Gulf War (Desert Storm: 1990–1991) and symptoms consistent with GWI as defined by the Centers for Disease Control and Prevention (CDC) and Kansas criteria for GWI. [23,24]. Subjects' ages ranged from 23 to 81 years (mean age = 58.9 years). For ME cases, the ratio of females to males was approximately 2 to 1 (64% and 36%, respectively); for GWI cases the ratio of males to females was approximately 2 to 1 (64% and 36%, respectively). Controls were of approximately equal proportions (57% male and 43% female).

The K.W. test was initially utilized to compare the individual cytokines concurrently for GWI and ME cases as well as healthy controls. Of the 77 cytokines analyzed, 48 (63%) differed for at least one of the three groups ($p \leqslant 0.05$), suggesting that the respective cytokine values did not originate from the same distribution (Supplemental data Table 1). We next utilized the M.W. test to compare the two groups of cases with each other and each group of cases with the control group (Supplemental data Table 1). When GWI and ME subjects were compared, 48 cytokines were observed to be significantly different ($p \leqslant 0.05$). Additionally, when ME cases were compared to healthy controls, 42 cytokines were observed to be differentially expressed, 17 of which were upregulated and 26 were downregulated (Supplemental data Table 2). This is in contrast to only 14 cytokines that differed between GWI cases and controls, 7 of which were upregulated and 7 were downregulated (Table 1). These observations suggest that, with respect to cytokines, GWI cases resemble healthy controls to a greater extent than they resemble ME cases.

3.2. Th1, Th2, and Th17 cytokine expression

Previous studies have reported that Th1, Th2, and Th17 cytokines, or combinations thereof, characterize GWI and ME. To

Table 1
Cytokines upregulated and downregulated in subjects with Gulf War illness (GWI) when compared to healthy controls.

Analyte	Group	Minimum	Maximum	Mean	Standard deviation	P value by M.W.
Cytokines upregulated in GWI subjects						
CCL11	CON	20.7	90.9	47.1	16.4	0.008
	GWI	22.2	252.8	72	48.4	
FGF	CON	3	98.5	20.7	17	0.026
	GWI	9.2	57.7	23.9	11.1	
IFN-γ	CON	2.8	50.4	15.4	10.3	0.003
	GWI	7	47.5	22.4	10.6	
IL-17A	CON	2.5	10.5	7.5	1.5	0.032
	GWI	0.8	277.8	13.6	44.8	
IL-17F	CON	1	143.5	17.9	30.8	0.001
	GWI	9.4	678.8	47.8	113.5	
IL-33	CON	8.2	2850.6	723.3	646	0.011
	GWI	56.5	4379.6	1112.2	883.4	
IL-5	CON	2.4	6.4	5.9	0.9	0.014
	GWI	0.1	24.5	6.4	3.4	
Cytokines downregulated in GWI subjects						
CCL5	CON	300.4	27934.9	5520.3	4290.5	0.031
	GWI	497.4	10415.4	4059.3	2015	
CXCL8	CON	6.4	3660.2	267.4	620.8	0.032
	GWI	2.2	1413.5	126.3	284	
IL-13	CON	8.1	11.3	9	1.3	0.001
	GWI	8.1	13.9	8.2	1	
IL-25	CON	0	4.7	2.4	2	0.04
	GWI	0	4.7	1.3	1.4	
IL-4	CON	0.1	2	1.1	0.6	0.041
	GWI	0.1	4.6	0.9	0.7	
IL-7	CON	1.4	11.8	5.5	3.2	0.011
	GWI	2.5	18.9	3.7	2.7	
TNF-α	CON	17.6	72.2	23.1	10.2	<0.0001
	GWI	5.7	20	19.3	3	

Table 2
Th1, Th2, and Th17 cytokines expression in subjects with GWI and ME.

Analyte	CON	ME	GWI	P value by Mann Whittney	
				ME vs CON	GWI vs CON
Th1 cytokines					
IFN-γ	15.4	32.4	22.4	0.001	0.003
IL-2	4.7	11.3	3.6	0.062	0.271
TNF-α	23.1	45.4	19.3	<0.0001	<0.0001
IL-12(p75)	20	32.7	21.9	<0.0001	0.246
CXCL8	267.4	191.9	126.3	<0.0001	0.032
IL-18	790.2	726.3	873.2	0.219	0.446
IL-12(p40)	290.1	238.3	309.9	0.089	0.914
Th2 cytokines					
IL-4	1.1	1.7	0.9	<0.0001	0.041
IL-13	9	10.7	8.2	<0.0001	0.001
IL-1β	7.9	14.4	6.6	<0.0001	0.133
IL-25	2.4	5	1.3	<0.0001	0.04
IL-10	10.2	31.7	10.8	0.017	0.223
IL-5	5.9	5.3	6.4	<0.0001	0.014
IL-6	4.3	12.4	3.6	<0.0001	0.202
IL-9	31.7	8	8.7	<0.0001	0.1
Th17 cytokines					
IL-17A	7.5	8.7	13.6	0.053	0.032
IL-17F	17.9	2.9	47.8	<0.0001	0.001
IL-21	30.1	44.5	36.2	0.918	0.081

explore this possibility, we organized these cytokines into three groups (Table 2). For subjects with ME, the Th1 cytokines IFN-γ, IL-2, and IL-12 (p75) were upregulated, while the Th2 cytokine IL-5 and IL-9 were downregulated. Paradoxically, the classical Th2 cytokines IL-4, IL-6, IL-10, IL-13 were also upregulated in subjects with ME. For subjects with GWI, the Th1 cytokine IFN-γ was upregulated; however, CXCL8 was slightly downregulated. Additionally, the Th2 cytokines IL-4, IL-13 and IL-25 were all significantly downregulated. For the Th17 cytokines, we observed IL-17F to be significantly downregulated in ME cases, while IL17A and IL17F were both significantly upregulated in GWI cases. Of the 48 cytokines that were differentially expressed in ME, 12 (25%) represented Th1, Th2, or Th17 cytokines. In contrast, of the 14 differentially expressed cytokines observed in GWI subjects (Table 2), 9 (64%) represented Th1, Th2, or Th17 cytokines.

3.3. Cytokine correlation analysis

In addition to T cells, other cells make many of the cytokines typically associated with a Th1, Th2 or Th17 shifts. For instance, the endogenous pyrogen TNF-α is primarily made by activated macrophages, but is also made by most nucleated cells including lymphocytes, fibroblasts and neurons [26,27]. Likewise, IL-6 is produced by activated macrophages as well as T cells and can act in a proinflammatory or antiinflammatory capacity [28]. Therefore, we conducted correlation analysis, in order to provide additional clues as to which cells produce these cytokines in our study groups. Our results suggest the Th1 cytokines strongly correlate in the ME population but substantially less so for the Th2 cytokines (Table 3). Additionally, a complete absence of correlation was observed for the Th17 cytokines in the ME group. In contrast to the ME group, only IL-2 and IL-12(p75) showed any significant correlation in GWI. Again, with respect to the GWI group, the Th1 cytokines showed a much weaker correlation and strikingly, TNF-α was negatively correlated with IFN-γ and IL-2, which is in contrast to the ME and control groups (Table 3). Also, the Th17 cytokines showed a moderately positive correlation in the GWI population. Interestingly, the correlation between IL-17A and IL-21 showed a positive correlation in contrast to that of the control population, which showed a negative correlation (Table 3).

In addition to the Th1, Th2 and Th17 cytokines, we conducted correlation analysis on the remaining analytes and observed almost perfect correlation ($R^2 \geqslant 0.90$) between a number of cytokines in the ME group (26 cytokines) but fewer in the GWI group (14 cytokines) (Supplemental data Table 3). Of particular interest, we observed almost perfect correlation between IL-7/IL-13, IL-7/FGF, and IL-7/TNF-α and between IL-3/IFN-α in the ME group. We also observed near perfect correlation between IL-1α/IL-3, IL-1α/IL-12(p40), IL-1α/IL-2 and IL-1α/IL-22 and between IL-1/IL-7 and IL-1/IL-13 in the GWI group.

3.4. Classification of cytokines by importance

Both GWI and ME are diseases with undefined etiology and both are often characterized by aberrant cytokine expression; however, the patterns of cytokine expression appear to be more complex than can be described by a standard Th1, Th2 or Th17 shift. With this in mind, we utilized the machine logic algorithm Random Forest (RF) to analyze our data set and potentially identify the most important cytokines that define these diseases. For this analysis, 500 random decision trees were built using six predictors for each node, and auto bootstrap out-of-bag sampling was employed to test the model. The 20 most significant cytokines for delineation of each group of subjects in order of decreasing importance were IL-7, IL-4, TNF-α, IL-13, IL-17F, IL-1, IL-5, IL-25, CXCL8, VEGF, CCL11, IL12(p75), IL-9, CFS3, IFN-γ, CCL4, IL-6, CCL2, CXCL1, and CXCL10 (Fig. 1). Using only serum cytokines,

Table 3
Correlation of cytokines in ME, GWI and controls (values given as R-squared).

Th1 cytokines ME					Th1 cytokines GWI					Th1 cytokines CON			
	IL-12p75	TNFα	IL-2			IL-12p75	TNFα	IL-2			IL-12p75	TNFα	IL-2
IFNγ	0.944	0.797	0.934		IFNγ	0.408	−0.495	0.424		IFNγ	0.305	0.346	0.339
IL-2	0.929	0.562			IL-2	0.724	−0.166			IL-2	0.837	0.802	
TNFα	0.758				TNFα	0.012				TNFα	0.534		

Th2 cytokines ME					Th2 cytokines GWI					Th2 cytokines CON				
	IL-9	IL-6	IL-5	IL-13		IL-9	IL-6	IL-5	IL-13		IL-9	IL-6	IL-5	IL-13
IL-4	0.065	0.282	0.561	0.679	IL-4	0.354	0.152	0.674	0.872	IL-4	−0.011	0.660	−0.742	0.878
IL-13	0.074	0.068	0.946		IL-13	0.249	0.16	0.896		IL-13	−0.121	0.680	−0.633	
IL-5	0.056	0.019			IL-5	0.306	0.117			IL-5	0.113	−0.462		
IL-6	−0.017				IL-6	0.013				IL-6	0.041			

Th17 cytokines ME			Th17 cytokines GWI			Th17 cytokines CON		
	IL-21	IL-17F		IL-21	IL-17F		IL-21	IL-17F
IL-17A	−0.016	−0.022	IL-17A	0.349	0.102	IL-17A	−0.544	−0.337
IL-17F	−0.036		IL-17F	0.592		IL-17F	0.815	

Table 4
Random Forest model statistics.

Class	N cases	N misclassified	Pct. error	Cost		
Out-of-bag testing						
Misclassification						
ME	67	5	7.46%	0.0746		
GWI	37	13	35.14%	0.3514		
CON	42	28	66.67%	0.6667		
Out-of-bag testing						
Prediction success						
Actual class	Total class	Percent correct	ME $N = 70$	GWI $N = 46$	CON $N = 30$	
ME	67	92.54%	62	1	4	
GWI	37	64.86%	1	24	12	
CON	42	33.33%	7	21	14	
Total	146					
Average		63.58%				
Overall% correct		68.49%				
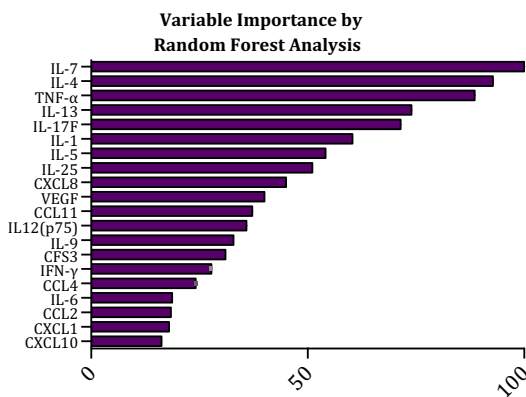
Fig. 1. Classification analysis of cytokine data using Random Forest. In order to identify which cytokines most accurately predict disease status of subjects with GWI, ME or controls, Random Forest analysis was implemented whereby 500 random trees were built and six predictors were used at each node. Auto-bootstrap out-of-bag sampling was used for testing the model.

we were able to achieve sensitivity of 92.5% for delineating ME; however, only 64.9% sensitivity was achieved when delineating GWI with 33.3% overall specificity (Table 4). These data indicate that using serum cytokines alone may not yield an effective diagnostic tool; however, it may provide important clues regarding the underlying pathology of the disease.

4. Discussion

Previous studies of GWI and ME often report that study subjects are characterized by abnormal numerical and functional leukocyte parameters. For example, when compared to healthy controls, NK cell enumeration and functionality have been reported to be abnormal in both diseases [7,29]. Additionally, atypical cytokine expression profiles are often reported in association with GWI and ME, although the results are often contradictory. For example, a distinct immune profile of attenuated Th1/Th17 and elevated Th2 responses was reported by Broderick et al., in subjects with ME [9]. However, in another study, Moss et al. observed upregulation of proinflammatory cytokines in the serum of ME cases, suggestive of a Th17 shift [30]. Likewise, cytokine profiling in GWI has been fraught by contradictory results, where predominant Th1 or Th2 immune responses have been reported [14,20,31]. Consequently, although differences in serum or plasma cytokines are well documented between cases and controls in both diseases, there is no consensus on a dominant cytokine expression profile for either disease. These conflicting findings may be a result of the heterogeneous nature of these diseases or perhaps a result of different methods of analysis or blood collection procedures. It is also likely that, at any given time, cytokine expression of an individual may change over time, complicating their use as a diagnostic marker.

In this report, we have presented a comprehensive analysis of 77 different cytokines, which to our knowledge represents the largest investigation of serum cytokines in GWI and ME subjects to date. Subjects' blood was collected using serum separator tubes and centrifuged immediately in order to isolate serum cytokines without the use of anticoagulants. By using this method, we have eliminated the possibility that the observed results were subject to leukocyte activation associated with anticoagulants such as heparin or assay interference associated chelating agents like ethylenediaminetetraacetic acid (EDTA).

In this study, we observed differences between cases and controls for 48 of the 77 cytokines investigated, using a confidence interval of 95%. Of the cytokines analyzed, 42 (54.5%) were found to be significantly different between ME cases and healthy controls. In contrast, only 14 cytokines (17.7%) were found to be significantly different between GWI cases and controls. Additionally, when comparing GWI and ME cases, 48 of 77 cytokines were differentially expressed. These data suggest that subjects with GWI and ME are unlikely to represent the same population.

Previous studies have suggested that subjects with GWI and ME may be characterized through the expression of either Th1 or Th2 cytokines. Upon activation, proliferating helper T cells may develop into effector T cells that are often classified as either Th1 or Th2 cells. Th1 immunity is directed against intracellular pathogens such as viruses and mycobacteria, whereas Th2 immunity is typically in response to extracellular pathogens such as fungi and helminths. For these reasons, the cytokines produced by these cells are also referred to as Th1- or Th2-type cytokines. The cytokine expression observed in this study with respect to ME cases, was largely inconsistent with a clear Th1- or Th2-type immune response. For instance, we observed an upregulation of IFN-γ ($p \leqslant 0.001$) and IL-12(p75) ($p \leqslant 0.001$) in the absence of an increase in IL-12(p40). These data are consistent with a classic Th1 response [32]. On the other hand, we also observed an upregulation of the IL-10 and IL-4, and when compared to healthy controls ($p \leqslant 0.0001$), suggestive of a Th2 response. These observations, in conjunction with our correlation analysis, suggest that the Th1 and Th2 cytokines observed in subjects with ME may originate from other immune cells in addition to T cells.

Our data more strongly supports a Th1/Th17 immune polarization in subjects with GWI. Serum cytokine analysis of these subjects showed an upregulation of the Th1 cytokine IFN-γ

($p \leqslant 0.003$) and the Th17 cytokines IL-17A ($p \leqslant 0.032$) and IL-17F ($p \leqslant 0.001$) and a concomitant downregulation of the Th2 cytokines IL-4 ($p \leqslant 0.014$) and IL-13 ($p \leqslant 0.001$) when compared to healthy controls. Exposures to such things as toxins, vaccines and unknown infectious agents have been suggested as potential triggers for GWI [5,6]. Several such triggers have been associated with a Th1/Th17 cytokine shift. For instance, Robbe et al. reported that the occupational exposure to agricultural dust was associated with upregulation of IL-17 and IFN-γ [33], and Harris and coworkers reported that human DCs upregulate IL-17 and IFN-γ in response to the bacteria *B. anthracis* [34]. Additionally, the cationic liposome adjuvant system CAF01, which is commonly used in such vaccines as the trivalent influenza vaccine, is reported to promote a strong and sustained Th1 and Th17 response [35]. Although we cannot say that any of these triggers contribute to GWI, the observed Th1/Th17 shift would be consistent with such triggers.

Little is known regarding the pathophysiology of GWI and ME; nevertheless, the source or class of cytokines produced in subjects with these diseases may provide important clues. Indeed, cytokine profiling has provided valuable knowledge regarding the pathogenesis of other diseases. For example, DeFuria et al. used cytokine profiling to identify the source of inflammatory cytokines associated with type 2 diabetes [36], and Swindle and coworkers utilized cytokine expression data to dissect the psoriatic transcriptome and identified the respective cellular contributions associated with this disease [37]. Additionally, Valeyev and colleagues showed that using a systems model approach; cytokine expression data could be used to provide a quantitative description of immune cell interactions in subjects with psoriasis [38]. In order to identify potential cytokines that may provide information regarding the pathogenesis of GWI and ME, we implemented the machine logic algorithm Random Forest (RF) to analyze our data set. The RF algorithm uses an ensemble of unpruned classification or regression trees produced through bootstrap sampling of the training data set and random feature selection in tree generation. Prediction was made by a majority vote of the predictions of the ensemble. The strength of the analysis was evaluated by an out-of-bag sampling without replacement of the original data. The RF is an attractive method since it handles both discrete and continuous data, it accommodates and compensates for missing data, and it is invariant to monotonic transformations of the input variables. The RF algorithm is uniquely suited for cytokine analysis in that it can handle highly skewed values well and weighs the contribution of each cytokine according to its relatedness with others. Using cytokine expression as input variables and subject status (i.e., GWI case, ME case, and control) as the outcome variable, we identified a group of cytokines that associated with disease status and, therefore, may contribute to the pathogenesis of the disease.

The five most significant cytokines identified by our model in decreasing order of importance were IL-7, IL-4, TNF-α, IL-13, and IL-17F. These cytokines were also identified by significant correlations in our analysis. IL-7 is a hematopoietic growth factor and is important for development, maturation and homeostasis of B, T, and NK cells. Stromal cells of the bone marrow and thymus are the primary source of IL-7; however, is it is also produced to a lesser extent by DCs, hepatocytes, and neurons, but not by lymphocytes [39]. Our data suggest that IL-7 is over-expressed in ME ($p \leqslant 0.001$) and under-expressed in GWI ($p \leqslant 0.01$) when compared to healthy controls. Previous studies suggest that the administration of exogenous of IL-7 in humans leads to the expansion of CD4$^+$ and CD8$^+$ T cells with a concomitant decrease of CD4$^+$ Tregs [40]. Other studies have shown that IL-7-treated animals have reduced numbers of T cells expressing the inhibitory molecules suppressor of cytokine signaling 3 (SOCS3) and

programmed cell death protein 1 (PD-1) [41]. Several studies suggest that ME is an inflammatory disease, and multiple reports of individuals with ME expressing autoantibodies [42,43] and the efficacious treatment of ME cases with the B-cell-depleting drug rituximab [44,45], suggest that some components of ME pathology may also overlap with those of autoimmunity. Aberrant expression of IL-7 and its receptor has been associated with several autoimmune diseases including inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis, and Sjögren's syndrome (Reviewed in [46]). The upregulation of IL-7 may help explain some of the clinical observations associated with ME such as the inflammatory component or the presentation of autoimmune-like symptoms. In that IL-7 is primarily produced in the bone marrow, but not by lymphocytes, transcriptional studies of bone marrow would be prudent; however, given the difficulty in collecting bone marrow biopsies, deciphering its involvement in these diseases may prove difficult. It is also likely that transcriptional profiling studies that only utilize whole blood will fail to identify an important component to the pathophysiology of these diseases.

Our analysis identified IL-4 and TNF-α as the second and third most important cytokines when delineating ME, GWI, and controls. Both IL-4 and TNF-α were over-expressed in ME ($p \leqslant 0.0001$) and under-expressed in GWI ($p \leqslant 0.04$ and $p \leqslant 0.0001$). IL-4 is a classic Th2 cytokine and promotes the differentiation of naïve helper T cells into Th2 cells. Once differentiated, the Th2 cells can produce additional IL-4 in a positive feedback control loop [47]. The chronic nature of these diseases suggests that Th2 cells are a likely source of serum IL-4; however, our correlation analysis suggests that they may not be the only source. During an inflammatory response, IL-4 production is often accompanied with IL-10 production, which is also upregulated in our ME subjects; however, no statistical difference was observed for IL-10 in GWI subjects. Interestingly, IL-13 was the next most important cytokine in our model. Expression of IL-13 was slightly downregulated in GWI and upregulated in ME. Our analysis showed that IL-13 and TNF-α expression was almost perfectly correlated ($R^2 = 0.918$). IL-13 is an antiinflammatory cytokine and its upregulation may be a response to counter the inflammatory effects of TNF-α. Lastly, our model identified IL-17F as the fifth most important cytokine in differentiating GWI cases, ME cases, and healthy controls. We observed IL-17F to be significantly downregulated in ME cases ($p \leqslant 0.0001$) and upregulated in GWI cases ($p \leqslant 0.001$). By increasing the production of inflammatory chemokines, IL-17 is a potent mediator of delayed-type responses and its expression promotes the recruitment of monocytes and neutrophils to the site of inflammation. IL-17F, in particular, is associated with respiratory pathology such as asthma [48].

Our analysis using RF suggests that any combination of the 77 cytokines analyzed in our study may not provide a stand-alone differential diagnosis of GWI and ME. Although the cytokine signature delineated ME cases with 92.5% efficiency, only 64.9% sensitivity was achieved when delineating GWI cases. Furthermore, specificity was 33.3% using cytokines only. Our ongoing research suggests that, by using a combination of cytokines and clinical parameters, we can far exceed the sensitivity and specificity of these results (data not shown). This observation further suggests that cytokines are useful when stratifying subjects into discrete subgroups. It also suggests that the "catch all" terms of GWI and ME may be overly broad. In light of the heterogeneous nature of these diseases, stratification into subgroups may be mandatory in order to make meaningful progress in understanding the pathophysiology of these diseases.

In conclusion, this study supports an involvement for Th1/Th17 cytokines in GWI and further identifies the cytokines IL-7, IL-4, TNF-α, IL-13, and IL-17F as potentially contributing to the

pathogenesis of GWI and ME. This knowledge may provide direction in the development of therapeutic treatments for these diseases.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2014.11.019.

References

[1] Carruthers BM, van de Sande MI, De Meirleir KL, Klimas NG, Broderick G, Mitchell T, et al. Myalgic encephalomyelitis: international consensus criteria. J Intern Med 2011;270:327–38.

[2] Nicolson GL, Nicolson NL. Gulf War illnesses: complex medical, scientific and political paradox. Med, Conflict, Survival 1998;14:156–65.

[3] De Meirleir K, Khaiboullina S, Fremont M, Hulstaert J, Rizvanov A, Palotas A, et al. Plasmacytoid dendritic cells in the duodenum of individuals diagnosed with myalgic encephalomyelitis are uniquely immunoreactive to antibodies to human endogenous retroviral proteins in vivo; 2013. p. 27.

[4] Buchwald D, Umali J, Pearlman T, Kith P, Ashley R, Wener M. Postinfectious chronic fatigue: a distinct syndrome? Clin Infect Dis: Off Publ Infect Dis Soc Am 1996;23:385–7.

[5] Iversen A, Chalder T, Wessely S. Gulf War illness: lessons from medically unexplained symptoms. Clin Psy Rev 2007;27:842–54.

[6] Fukuda K, Nisenbaum R, Stewart G, Thompson WW, Robin L, Washko RM, et al. Chronic multisymptom illness affecting Air Force veterans of the Gulf War. JAMA, J Am Med Assoc 1998;280:981–8.

[7] Gupta S, Vayuvegula B. A comprehensive immunological analysis in chronic fatigue syndrome. Scand J Immunol 1991;33:319–27.

[8] Whiteside TL, Friberg D. Natural killer cells and natural killer cell activity in chronic fatigue syndrome. Am J Med 1998;105:27S–34S.

[9] Broderick G, Fuite J, Kreitz A, Vernon SD, Klimas N, Fletcher MA. A formal analysis of cytokine networks in chronic fatigue syndrome. Brain Behav Immun 2010;24:1209–17.

[10] Tomoda A, Joudoi T, Rabab el M, Matsumoto T, Park TH, Miike T. Cytokine production and modulation: comparison of patients with chronic fatigue syndrome and normal controls. Psychiatry Res 2005;134:101–4.

[11] Patarca-Montero R, Antoni M, Fletcher MA, Klimas NG. Cytokine and other immunologic markers in chronic fatigue syndrome and their relation to neuropsychological factors. Appl Neuropsy 2001;8:51–64.

[12] Chao CC, Janoff EN, Hu SX, Thomas K, Gallagher M, Tsang M, et al. Altered cytokine release in peripheral blood mononuclear cell cultures from patients with the chronic fatigue syndrome. Cytokine 1991;3:292–8.

[13] Natelson BH, Weaver SA, Tseng CL, Ottenweller JE. Spinal fluid abnormalities in patients with chronic fatigue syndrome. Clin Diagn Lab Immunol 2005;12:52–5.

[14] Zhang Q, Zhou XD, Denny T, Ottenweller JE, Lange G, LaManca JJ, et al. Changes in immune parameters seen in Gulf War veterans but not in civilians with chronic fatigue syndrome. Clin Diagn Lab Immunol 1999;6:6–13.

[15] Maes M, Twisk FN, Johnson C. Myalgic Encephalomyelitis (ME), Chronic Fatigue Syndrome (CFS), and Chronic Fatigue (CF) are distinguished accurately: results of supervised learning techniques applied on clinical and inflammatory data. Psychiatry Res 2012;200:754–60.

[16] Maes M, Twisk FN, Kubera M, Ringel K. Evidence for inflammation and activation of cell-mediated immunity in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS): increased interleukin-1, tumor necrosis factor-alpha, PMN-elastase, lysozyme and neopterin. J Affect Disord 2012;136:933–9.

[17] Fletcher MA, Zeng XR, Barnes Z, Levis S, Klimas NG. Plasma cytokines in women with chronic fatigue syndrome. J Trans Med 2009;7:96.

[18] Smylie AL, Broderick G, Fernandes H, Razdan S, Barnes Z, Collado F, et al. A comparison of sex-specific immune signatures in Gulf War illness and chronic fatigue syndrome. BMC Immunol 2013;14:29.

[19] Skowera A, Cleare A, Blair D, Bevis L, Wessely SC, Peakman M. High levels of type 2 cytokine-producing cells in chronic fatigue syndrome. Clin Exp Immunol 2004;135:294–302.

[20] Skowera A, Hotopf M, Sawicka E, Varela-Calvino R, Unwin C, Nikolaou V, et al. Cellular immune activation in Gulf War veterans. J Clin Immunol 2004;24:66–73.

[21] Carruthers B, Jain AK, De Meirleir K, Peterson D, Klimas N, Lerner A, et al. Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: clinical working case definition, diagnostic and treatment protocols. J Chron Fatigue Synd 2003;11:1–12.

[22] Fukuda K, Straus SE, Hickie I, Sharpe MC, Dobbins JG, Komaroff A. The chronic fatigue syndrome: a comprehensive approach to its definition and study. International chronic fatigue syndrome study group. Ann Intern Med 1994;121:953–9.

[23] Centers for Disease C, Prevention. Unexplained illness among Persian Gulf War veterans in an Air National Guard Unit: preliminary report – August 1990-March 1995. MMWR Morbidity and mortality weekly report. 1995; 44: 443–7.

[24] Steele L. Prevalence and patterns of Gulf War illness in Kansas veterans: association of symptoms with characteristics of person, place, and time of military service. Am J Epidemiol 2000;152:992–1002.

[25] Breiman L. Random Forests. Mach Learn 2001;45:5–32.

[26] Ware CF, Crowe PD, Grayson MH, Androlewicz MJ, Browning JL. Expression of surface lymphotoxin and tumor necrosis factor on activated T, B, and natural killer cells. J Immunol 1992;149:3881–8.

[27] Grivennikov SI, Tumanov AV, Liepinsh DJ, Kruglov AA, Marakusha BI, Shakhov AN, et al. Distinct and nonredundant in vivo functions of TNF produced by t cells and macrophages/neutrophils: protective and deleterious effects. Immunity 2005;22:93–104.

[28] Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 2011;1813:878–88.

[29] Whistler T, Fletcher MA, Lonergan W, Zeng XR, Lin JM, Laperriere A, et al. Impaired immune function in Gulf War illness. BMC Med Genomics 2009;2:12.

[30] Moss RB, Mercandetti A, Vojdani A. TNF-alpha and chronic fatigue syndrome. J Clin Immunol 1999;19:314–6.

[31] Rook GA, Zumla A. Gulf War syndrome: is it due to a systemic shift in cytokine balance towards a Th2 profile? Lancet 1997;349:1831–3.

[32] Abdi K. IL-12: the role of p40 versus p75. Scand J Immunol 2002;56:1–11.

[33] Robbe P, Spierenburg EA, Draijer C, Brandsma CA, Telenga E, van Oosterhout AJ, et al. Shifted T-cell polarisation after agricultural dust exposure in mice and men. Thorax 2014;69:630–7.

[34] Harris KM, Ramachandran G, Basu S, Rollins S, Mann D, Cross AS. The IL-23/Th17 axis is involved in the adaptive immune response to *Bacillus anthracis* in humans. Eur J Immunol 2014;44:752–62.

[35] Rosenkrands I, Vingsbo-Lundberg C, Bundgaard TJ, Lindenstrom T, Enouf V, van der Werf S, et al. Enhanced humoral and cell-mediated immune responses after immunization with trivalent influenza vaccine adjuvanted with cationic liposomes. Vaccine 2011;29:6283–91.

[36] DeFuria J, Belkina AC, Jagannathan-Bogdan M, Snyder-Cappione J, Carr JD, Nersesova YR, et al. B cells promote inflammation in obesity and type 2 diabetes through regulation of T-cell function and an inflammatory cytokine profile. Proc Natl Acad Sci USA 2013;110:5133–8.

[37] Swindell WR, Johnston A, Voorhees JJ, Elder JT, Gudjonsson JE. Dissecting the psoriasis transcriptome: inflammatory- and cytokine-driven gene expression in lesions from 163 patients. BMC Genomics 2013;14:527.

[38] Valeyev NV, Hundhausen C, Umezawa Y, Kotov NV, Williams G, Clop A, et al. A systems model for immune cell interactions unravels the mechanism of inflammation in human skin. PLoS Comput Biol 2010;6:e1001024.

[39] Fry TJ, Mackall CL. Interleukin-7: from bench to clinic. Blood 2002;99:3892–904.

[40] Rosenberg SA, Sportes C, Ahmadzadeh M, Fry TJ, Ngo LT, Schwarz SL, et al. IL-7 administration to humans leads to expansion of CD8+ and CD4+ cells but a relative decrease of CD4+ T-regulatory cells. J Immunother 2006;29:313–9.

[41] Pellegrini M, Calzascia T, Toe JG, Preston SP, Lin AE, Elford AR, et al. IL-7 engages multiple mechanisms to overcome chronic viral infection and limit organ pathology. Cell 2011;144:601–13.

[42] Konstantinov K, von Mikecz A, Buchwald D, Jones J, Gerace L, Tan EM. Autoantibodies to nuclear envelope antigens in chronic fatigue syndrome. J Clin Investig 1996;98:1888–96.

[43] von Mikecz A, Konstantinov K, Buchwald DS, Gerace L, Tan EM. High frequency of autoantibodies to insoluble cellular antigens in patients with chronic fatigue syndrome. Arthritis Rheum 1997;40:295–305.

[44] Fluge O, Bruland O, Risa K, Storstein A, Kristoffersen EK, Sapkota D, et al. Benefit from B-lymphocyte depletion using the anti-CD20 antibody rituximab in chronic fatigue syndrome. A double-blind and placebo-controlled study. PLoS ONE 2011;6:e26358.

[45] Fluge O, Mella O. Clinical impact of B-cell depletion with the anti-CD20 antibody rituximab in chronic fatigue syndrome: a preliminary case series. BMC Neurol 2009;9:28.

[46] Dooms H. Interleukin-7: fuel for the autoimmune attack. J Autoimmun 2013;45:40–8.

[47] Oliphant CJ, Barlow JL, McKenzie AN. Insights into the initiation of type 2 immune responses. Immunology 2011;134:378–85.

[48] Iwakura Y, Nakae S, Saijo S, Ishigame H. The roles of IL-17A in inflammatory immune responses and host defense against pathogens. Immunol Rev 2008;226:57–79.