Immune transcriptomic changes in Australian Gulf War veterans

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Abstract

Background

Gulf War Illness (GWI) is a chronic multisystemic illness found in one-third of Gulf War Veterans. The aetiology of GWI is elusive; however, is strongly associated with exposure to multiple toxic agents, environmental exposures, and prophylactic medications or vaccinations. In the literature, disruption of the immune system and the presence of inflammation have been reported in GWI. In this novel study, we report gene expression-based analysis of a panel of 785 immune function related gene markers in GWI.

Method

Ribonucleic acid (RNA) was extracted from peripheral blood mononuclear cells (PBMCs) isolated from n = 20 Australian GWI (CDC Case Definition and Kansas Criteria, 54.4 ± 0.74 years), and n = 15 healthy control (HC, 47.47 ± 2.91 years) participants. All participants were sex-matched (100% male). RNA gene expression was quantified using the NanoString® nCounter Immune Exhaustion panel and analysed using Rosalind Bio and IPA.

Results

Thirty-three differentially expressed genes were identified, of which 21 were upregulated and 12 were downregulated in the GWI cohort. Upregulated genes included *SIGLEC1, BPI, MMP9, RSAD2, IFIT1/2, CEACAM1/3* and were associated with metabolic and cellular stress responses, while downregulated genes were associated with T cell receptor regions and humoral immune responses. Downregulated genes included *TRDV3, IGHG1, TRGV4, TRDV1/4,* and *IL7*. Gene set analysis revealed associations between gene expression and type I interferon signalling, natural killer receptors, T cell receptors, and tumour necrosis factor signalling. Pathway analysis

revealed the role of differentially expressed genes in neutrophil signalling and degranulation, toll-like receptor cascades, and the role of lipids/lipid rafts in infection.

Conclusion

This investigation elucidates the potential role of immune dysregulation underlying GWI, emphasising the importance of immune exhaustion pathways in disease progression. Further investigations in a larger cohort may further elucidate or confirm these identified markers for potential screening or therapeutic applications in GWI.

Introduction

Between August 1990 and February 1991, a coalition of 41 countries and approximately a million veterans participated in the Gulf War (GW), including 1,800 Australians. Following the first deployment, several epidemiological studies reported complex multi-systemic symptoms in veterans of the GW. The presence of chronic symptoms including fatigue, sleep disturbances, respiratory and epithelial complaints, neurocognitive disturbances, and body pain is referred to as GW Syndrome/Illness (GWI), a phenomenon categorised under the umbrella term chronic multisymptom illness [1]. GWI affects an estimated 25–32% of GW veterans (GWV) deployed to the GW in 1990–1991 [2]. Epidemiological reports in Australian GWV are consistent with research conducted in the United States (U.S.), United Kingdom (U.K.), Canadian, and French veterans reporting [3].

The pathomechanism of GWI remains elusive, as a consequence, there is no diagnostic test nor evidenced-based treatment available. Furthermore, the aetiology of GWI is not completely understood. Current evidence supports the hypothesis that a combination of toxic chemical and environmental agents, including insecticides, smoke from oil-well fires, pyridostigmine bromide (PB) result in a veteran developing GWI [4]. These toxic environmental and chemical agents are found to be statistically associated with immune system dysfunction [5,6]. While intracellular mechanisms remain diverse, agents including organophosphates and carbamate insecticides and PB, used prophylactically by veterans, as well as products from oil well fires, including particulate matter, heavy metals and polycyclic aromatic hydrocarbons are linked with chronic inflammation, oxidative stress and neuronal damage [7–10]. Therefore, demonstrating that no single exposure results in the occurrence of GWI.

One mechanism may include the inhibition of acetylcholinesterase by insecticides and PB resulting in an accumulation of acetylcholine which consequentially influences cellular metabolism and immunological functions, including reactive oxygen species (ROS), chronic inflammation, impaired cytotoxic pathways, cytokine production and immune cell activation [7–10]. As an example, an investigation into the effect of the pesticide permethrin with PB on a GWI mice model reported increased activation of both peripheral and brain adaptive immune responses [11]. Further, the accumulation of acetylcholine is further linked with oxidative stress. In an experimental GWI mice model, impaired cellular metabolism promoting ROS have been reported in

association with immune dysregulation [12]. This is further evidenced in diverse immunological studies reporting lympho-cyte disturbances, altered lymphocyte subsets, interleukin (IL) and cytokine production, and production of antibodies are modified in veterans with GWI [13–16].

As many veterans continue to be affected in the decades following their return from the GW, research into the multisymptomatic pathomechanism of GWI is critical to avoid declining health in an aging population. Given the impact of immune responses linked to GWI symptomology, genetic variability that predisposes persistent inflam-matory/immune alterations have been investigated [17]. Investigations into immune abnormalities provide valuable insights that may inform future biomedical research resulting in potential diagnostic tools and therapeutic interven-tions. This investigation aimed to elucidate transcriptome changes associated with immune exhaustion in Australian veterans with GWI.

Method

Participants

Australian GWV with multisystem symptoms and diagnosed with GWI were recruited with the assistance of the Gulf War Illness Association of Australia. Veterans with GWI fulfilled the CDC Case Definition [18] and Kansas Criteria [19] for GWI. Healthy controls (HC) reported an absence of disease and/or chronic diagnoses. Participants were screened according to their medical history and symptom presentation and were asked to report on quality of life measurements, including the 36-item short form health survey (SF-36) and World Health Organization Disability Assessment Schedule (WHODAS). All participants were males, aged between 18 and 65 years and non-smokers. Participants were not included in this current study if they reported a history of alcohol abuse, cardiovascular dis-ease, thyroid disease, malignancies, insomnia, and another condition that may account for their symptoms. This investigation was approved by the Griffith University Human Research Ethics Committee (GU/2022/666). Research involving human research participants was performed per the Declaration of Helsinki and written consent was pro-vided by all individuals prior to participation.

Sample collection and preparation

Between 20–40 ml of whole blood was collected from each participant into ethylenediaminetetraacetic acid (EDTA) tubes via venepuncture at collection locations across Southeast Queensland and Northeast New South Wales from April 2022 to April 2024. All samples were collected between the hours of 7:00AM and 11:00AM from non-fasted participants. Four ml of EDTA whole blood was used for full blood count analysis.

Anonymised samples were delivered to the National Centre for Neuroimmunology and Emerging Diseases laboratory within four hours of collection. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation using Ficoll (GE Healthcare, Uppsala, Sweden) as previously described [20]. PBMCs were stained with trypan blue (Invitrogen, Carlsbad, CA, USA) to determine cell count and viability. PBMCs were resuspended in fetal bovine serum (FBS) (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% dimethyl sulfoxide (DMSO) and stored at -80°C until ribonucleic acid (RNA) extraction as previously described [21].

Frozen PBMCs were thawed and immediately pelleted by centrifugation in September 2024. Total RNA was isolated from PBMC pellets ($5–10 \times 10^6$ cells) using either the Trizol method (n = 2 samples) or a RNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer instructions. The concentration and quality of the RNA were measured using Nan-oDrop ND-1000 spectrophotometer (Thermo Scientific, Massachusetts, US). RNA purity values were recorded with the mean and standard deviation being 1.99 ± 0.10 for 260/280 and 1.33 ± 0.43 for 260/230. While contaminants appeared present according to 260/230 values, all samples passed RNA binding density. The quality of the samples was confirmed using LabChip RNA Standard Sense Assay. All RNA samples returned a quality score above 8.9.

RNA expression and NanoString®

RNA expression analysis was determined using the NanoString® nCounter Immune Exhaustion gene expression panel according to manufacturer's instructions (NanoString Technologies, Seattle, WA, USA). A full list of investigated genes and their functional themes can be found in S1 Table. Batches of 12 separate samples were prepared according to the manufacturer's instructions.

Raw gene expression data was normalised against positive and negative controls. Normalisation and analyses were performed using Rosalind Bio (San Diego, CA, USA) and the following housekeeping genes *ABCF1, ALAS1, EEF1G, G6PD, GAPDH, GUSB, HPRT1, OAZ1, POLR1B, POLR2A, PPIA, RPL19, SDHA, TBP,* and *TUBB* (S1 Table). All raw gene expression data, normalised counts and quality check outputs cancan be found in S1 Table. Differential expression (fold change (FC) >1.5 or <−1.5 and a P-value <0.05) is reported between GWI with HC. Ingenuity Pathway Analysis (IPA) (Qiagen Digital Insights, California, USA) was used to interpret differential RNA expression in biological pathways and networks using algorithms developed for use by [22].

Analysis

The normality of participant data was determined using the Shapiro-Wilk test. Normally distributed continuous data was compared using the independent student's T test and non-normally distributed continuous data was compared using the Mann-Whitney U test. Age, body mass index (BMI), full blood count analysis, and quality of life measures are presented as mean ± standard error of mean (SEM) unless otherwise stated. Remaining participant demographics, including highest level of education and employment status were compared using the Chi-Square test and the Fisher's exact test. The effect of age and BMI on gene expression between cohorts was investigated using a general linear multivariate regression model. Participant data was analysed using SPSS (version 27) and GraphPad Prism (version 10). Significance is set at $p < 0.05$. Adjusted (Adj.) p-values are provided unless otherwise stated.

Results

Participants

This current study included n = 15 HC and n = 20 GWI. Participants with GWI were significantly older and reported higher BMI compared with HC (p = 0.013). There were no significant differences between participant cohorts in terms of the highest level of education achieved. There was a significant difference in the status of employment between cohorts (p = 0.027). Full blood count was determined for all participants and no significant differences were found between cohorts. All participants with GWI reported significantly lower SF-36 scores across all domains and higher scores of disability in all domains compared with HC. All clinical characteristics and demographics of participants are summarised in Tables 1 and 2.

The prevalence of symptoms reported by GWI participants is reported in Table 3. The most commonly reported symptoms included cognitive disturbances, pain, sleep, and neurosensory disturbances (94.7% of cohort).

Differential gene expression

Of the 785 genes that are included in the NanoString Immune Exhaustion Panel, 601 genes passed background thresholding and normalisation. Differential expression of genes was filtered according to log fold change parameters −1.5 to 1.5 and a p-value of 0.05, resulting in the selection of 33 genes in GWI (Table 4 & Fig 1). Of the 33 selected genes, 21 were upregulated, and 12 were downregulated. Downregulated genes included *TRDV3 (T cell receptor delta variable 3)*, *IGHG1 (immunoglobulin (IG) heavy constant gamma 1)* and *TRDV4* ($\log_2$FC = −1.664, p = 0.004; $\log_2$FC = −1.438, p = 0.035; and $\log_2$FC = −1.20, p = 0.04, respectively). Of the upregulated genes *SIGLEC1 (sialic acid binding Id-like lectin)* and *BPI (bactericidal/permeability increasing protein)* had the highest degree of change with $\log_2$FC = 1.96 (p = 0.002), and $\log_2$FC = 1.65 (p = 0.004), respectively. The full dataset outputs can be found in S2 Table.

Table 1. Participant demographics.

	HC N(%)	GWI N(%)	P-value
Education n (%)			0.110
Primary School	0 (0.0)	0 (0.0)	
High School	3 (20.0)	7 (35.0)	
Undergraduate	2 (13.3)	8 (40.0)	
Postgraduate	7 (46.7)	3 (15.0)	
Other	3 (20.0)	2 (10.0)	
Employment n (%)			**0.027**
Full Time	11 (73.3)	9 (45.0)	
Part Time	2 (13.3)	0 (0.0)	
Casual	1 (6.7)	1 (5.0)	
Unemployed (other)	1 (6.7)	9 (45.0)	
Unemployed illness	0 (0.0)	1 (5.0)	
Sex n (%)			
Male	15 (100%)	20 (100.0%)	

Categorical variables compared using chi-square test. Data presented as n (%). *Abbreviations: HC, healthy control; GWI, Gulf War Illness.*

https://doi.org/10.1371/journal.pone.0338198.t001

Gene set analysis

The change in regulation within each gene set relative to the baseline was described using gene set analysis (GSA), both undirected enrichment score (UES) and directed enrichment score (DES) for the top 10 gene sets are presented in Table 5. GSA was obtained from Rosalind Bio. Differentially expressed genes in GWI were associated with Type I Interferon (UES = 1.7835, DES = 1.7386), NK receptors (UES = 1.6857, DES = −0.1878), fatty acid metabolism (UES = 1.4838, DES = −1.0185), tumour necrosis factor (TNF) signalling (UES = 1.3493, DES = 0.9285), and T cell receptor (UES = 1.319, DES = −0.7643). The full dataset outputs can be found in S2 Table.

Cell type abundance

The abundance of cell populations was calculated according to the expression of cell marker genes using Rosalind Bio. Hierarchical cluster analysis observations demonstrate similar distributions within cohorts for major immune cell populations (Fig 2). While not significant, veterans with GWI had a slightly higher mean abundance of T cells marker genes compared with HC and was accompanied by lower Treg cells. Neutrophils marker genes were also found to be lower in GWI relative to HC; however, this was not significant. Abundance scores for cell types are shown in S3 Table.

Pathways and disease functions

A summary of IPA analysis, including the top five biological functions and canonical pathways are presented in Table 6. The top five biological functions in GWI include childhood-onset systemic lupus erythematosus (SLE), ATAD3A-related type I interferonpathy, SLE, activation of leukocytes, and antineutrophil cytoplasmic antibody associated vasculitis (all p < 0.0001). *RSAD2* and *SIGLEC1* were consistently reported across all biological functions. The top five canonical pathways in GWI include neutrophil extracellular trap signalling pathway (p = 0.0023), neutrophil degranulation (p = 0.0031), role of lipids/lipid rafts in influenza (p = 0.0045), chaperone mediated autophagy signalling pathway (p = 0.0055) and toll-like receptor cascades (p = 0.006). The complete pathways and disease functions output can be found in S4 Table.

Table 2. Participant demographics, full blood analysis and quality of life.

	HC			GWI			P-value
	Mean	SEM	SD	Mean	SEM	SD	
Age	47.47	2.91	11.27	54.4	0.74	3.29	**0.013**
BMI	25.96	1.09	4.23	31.44	1.62	7.23	**0.013**
Full blood count analysis							
WCC (x10^9/L)	6.11	0.31	1.19	6.78	0.33	1.47	0.158
Lymphocyte (x10^9/L) [a]	1.88	0.19	0.77	2.03	0.14	0.63	0.214
Neutrophils (x10^9/L)	3.49	0.20	0.79	3.98	0.26	1.15	0.163
Monocytes (x10^9/L)	0.51	0.05	0.18	0.56	0.03	0.15	0.287
Eosinophils (x10^9/L) [a]	0.19	0.04	0.14	0.16	0.02	0.09	0.856
Basophils (x10^9/L)	0.05	0.01	0.02	0.05	0.004	0.02	0.924
Platelets (x10^9/L)	260.13	10.97	42.49	263.55	14.91	66.66	0.863
RCC (x10^{12}/L)	5.08	0.08	0.32	5.07	0.09	0.39	0.932
Haematocrit	0.44	0.01	0.02	0.45	0.01	0.03	0.082
Haemoglobin (g/L)	149.07	2.24	8.68	151.10	2.30	10.31	0.542
SF-36							
General Health	71.38	4.60	17.81	37.25	4.12	18.44	**<0.001**
Physical Functioning[a]	96.0	2.02	7.83	57.50	5.99	26.83	**<0.001**
Role Physical	82.92	9.46	36.63	45.31	6.44	28.81	**0.002**
Role Emotional	95.56	2.68	10.38	49.99	7.13	31.88	**<0.001**
Pain[a]	90.67	3.40	13.17	38.0	4.54	20.32	**<0.001**
Mental Health	83.67	3.57	13.82	47.50	5.37	24.03	**<0.001**
Vitality	75.83	3.39	13.12	40.63	3.06	13.68	**<0.001**
Social Functioning	97.50	1.81	7.01	43.75	6.87	30.75	**<0.001**
WHO DAS							
Understanding & communication	8.06	3.56	13.78	39.79	5.17	23.12	**<0.001**
Mobility	2.33	1.61	6.23	33.0	5.39	24.08	**<0.001**
Self-care[a]	0.83	0.83	3.23	15.0	3.37	15.09	**<0.001**
Relationships	5.42	2.58	9.98	45.0	5.82	26.02	**<0.001**
Life activities	2.08	1.45	5.62	36.56	5.29	23.67	**<0.001**
Participation in society	5.21	2.77	10.74	44.37	6.26	28.01	**<0.001**

Continuous variables compared using Mann Whitney U test or T test. [a] Denotes continuous variables compared using Mann Whitney U test. The WHO DAS domain for participation in work/school was omitted given the high number of participants reporting unemployment. Data presented as mean, SEM and SD. *Abbreviations: HC, healthy control; GWI, Gulf War Illness; BMI, body mass index; SD, standard deviation; SEM, standard error of mean; WCC, white cell count; RCC, red cell count; SF-36, 36 item short-form health survey; WHO, World Health Organization; DAS, disability assessment schedule.*

https://doi.org/10.1371/journal.pone.0338198.t002

Network analysis

Interaction network analysis was performed using IPA. This analysis demonstrates the interactions between molecules and the dataset imported. One network was exported, with a score of 16 (Fig 3). Analysis of this network, consisting of five focus molecules, was associated with cell-to-cell signalling and interactions, haematological system development and function, and immune cell trafficking. Focus molecules identified were *BPI* (p = 0.004), *MMP9* (p = 0.0004), *RSAD2* (p = 0.004), *SIGLEC1* (p = 0.002), and *TRDV3* (p = 0.005). Top upstream regulators were identified to be ATAD3A (p < 0.0001), TNF (family, p < 0.0001), RNY3 (p < 0.0001), SOD1 (p < 0.0001) and CSF1 (p < 0.0001). Molecules in causal

Table 3. Symptom prevalence in Australian GWI participants.

Symptom	n (%)
Post-exertional malaise	17 (89.5)
Cognitive disturbances	18 (94.7)
Pain	18 (94.7)
Sleep	18 (94.7)
Neurosensory, perceptual, and motor disturbances	18 (94.7)
Immune	9 (47.4)
Respiratory	9 (47.4)
Gastrointestinal	15 (78.9)
Urinary disturbances	11 (57.9)
Cardiovascular manifestations	11 (57.9)
Thermoregulatory disturbances	11 (57.9)

Data presented as n (%) for those reporting experiencing the symptom. Missing data n = 1. *Abbreviations: GWI, Gulf War Illness; n, sample number.*

https://doi.org/10.1371/journal.pone.0338198.t003

network identified ATAD3A (p < 0.0001), RNY3 (p < 0.0001), Jak (family) (p < 0.0001), and type I IFN genes (p < 0.0001). Network analysis outputs can be found in S4 Table.

Discussion

This novel study investigates altered gene expression related to immune function in Australian veterans with GWI compared with HC. Briefly, 33 differentially expressed genes were identified, 21 of which were upregulated and 12 were downregulated. Of the differentially expressed genes, *TRDV3*, *IGHG1*, *TRGV4*, *TRDV1*, and *TRDV2* returned as the top five downregulated, while *SIGLEC1, BPI, MMP9, RSAD2,* and *CEACAM1* were the top five upregulated genes. To the authors knowledge this present research is the first to conduct an analysis on immune exhaustion and inflammation markers simultaneously using NanoString Technology in this cohort.

Previous studies have investigated genetic markers in GWI with the identification of nerve agent susceptible genes including *PON1* (paraoxonase-1) and *BChE* (butyrylcholinesterase) [23,24] as well as the neurodegeneration gene *APOE* (apolipoprotein) [25]. Meanwhile, immune profiling has identified altered expression of inflammatory markers including *IL-1β, TNFα, MMP-2, CCL12,* and *EGF*, some of which aligning with the present investigation [26]. A previous study employing logistic regression modelling created a prediction model of GWI risk associated with genetic variability in *TGF* (rs1800469, p = 0.009), *IL6R* (rs8192284, p = 0.004) and *TLR4* (rs4986791, p = 0.013) [17]. While variability within these mentioned genes was not identified in the present study, toll-like receptor cascades and IL signalling were significantly associated with the top differential genes identified in the present manuscript. Further research has also reported an associated with human leukocyte antigen (HLA) allele DRB1*13:02 [27]. Previous research and the present study identified potential markers worthy of further investigation to elucidate the role of immune disturbances in the pathomechanism of GWI.

TCR genes (*TRDV3*, *TRDV1*, *TRDV2*, *TRGV4* and *TRGC1*) were downregulated in Australian veterans with GWI compared with HC, suggesting potential consequences that result in altered downstream T cell activity and cytotoxic function. This is supported by the abovementioned GSA data whereby TCR signalling returned a negative DES, indicating a functional reduction. A downregulation of TCRs will impair T cell responsiveness to antigen stimulation through impaired T cell to target cell interactions and a decrease in downstream intracellular signalling cascades [28]. TCR downregulation accompanied by reduced IL-2 signalling, demonstrated through a negative DES above, further supports the suggestion of impaired T cell survival and proliferation in GWI [29].

Table 4. Differential gene expression.

Gene	Description	log2FC	P-value
Downregulated			
TRDV3	T cell receptor delta variable 3	−1.6643	0.0049
IGHG1	Immunoglobulin heavy constant gamma 1	−1.4380	0.0345
TRGV4	T cell receptor gamma variable 4	−1.2019	0.0496
TRDV1	T cell receptor delta variable 1	−1.1313	0.0018
TRDV2	T cell receptor delta variable 2	−1.1312	0.0269
IL7	Interleukin 7	−0.8934	0.0170
IGHV4–59	Immunoglobulin heavy variable 4–59	−0.7757	0.0351
EHHADH	3-hydroxyacyl CoA dehydrogenase	−0.7612	0.0107
IDO1	Indoleamine 2,3-dioxygenase 1	−0.7149	0.0473
CXCR6	chemokine (C-X-C motif) receptor 6	−0.7101	0.0160
TRGC1	T cell receptor gamma constant 1	−0.7017	0.0098
SESN2	Sestrin 2	−0.6490	0.0221
Upregulated			
SIGLEC1	Sialic acid binding Ig-like lectin 1, sialoadhesion	1.9591	0.0022
BPI	Bactericidal/permeability-increasing protein	1.6511	0.0045
MMP9	Matrix metallopeptidase 9	1.5475	0.0004
RSAD2	Radical S-adenosyl methionine domain containing 2	1.5471	0.0037
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	1.4122	0.0022
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	1.3933	0.0111
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	1.3519	0.0075
CXCL1/2/3	Chemokine (C-C motif) ligand 1/2/3	1.3366	0.0292
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1.3063	0.0287
CEACAM3	Carcinoembryonic antigen-related cell adhesion molecule 3	1.2773	0.0045
ITGB3	Integrin beta 3 (platelet glycoprotein IIIa antigen CD61)	1.2059	0.0019
EGF	Epidermal growth factor	1.0986	0.0251
LTBP1	Latent transforming growth factor beta binding protein 1	1.0477	0.0074
GREM2	Gremlin 2 DAN family BMP antagonist	0.9805	0.0438
OAS3	2'-5'-oligoadenylate synthetase 3 (100kDa)	0.9389	0.0129
IL1B	Interleukin 1 beta	0.9364	0.0483
ELOVL7	ELOVL fatty acid elongase 7	0.8905	0.0079
MX1	MX dynamin-like GTPase 1	0.8547	0.0191
TNFAIP3	Tumor necrosis factor alpha-induced protein 3	0.7483	0.0209
FCAR	Fc fragment of IgA receptor	0.6453	0.0153
MX2	MX dynamin-like GTPase 2	0.5934	0.0115

Data extracted from Rosalind Bio. Descriptions extracted from the National Institutes of Health (NIH) National Library of Medicine gene database. Abbreviations: FC, fold change; TRDV3, T cell receptor delta variable 3; IGHG1, Immunoglobulin heavy constant gamma 1; TRDV2, T cell receptor delta variable 2; TPSAB1/B2, tryptase alpha/beta 1; TRGV4, T cell receptor gamma variable 4; TRDV1, T cell receptor delta variable 1; IL7, Interleukin 7; IGHV4−59, immunoglobulin heavy variable 4−59; IDO1, indoleamine 2 3-dioxygenase 1; EHHDAH, enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase; CXCR6 chemokine (C-X-C motif) receptor 6; TRGC1, T cell receptor gamma constant 1; SESN2, sestrin 2; SIGLEC, sialic acid binding Ig-like 1; BPI, bactericidal/permeability-increasing protein; RSAD2, radical S-adenosyl methionine domain containing 2; MMP9, Matrix metallopeptidase 9; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; CEACAM1, Carcinoembryonic antigen-related cell adhesion molecule 1; IFIT3, interferon-induced protein with tetratricopeptide repeats 3; CXCL1/2/3, CXCL1, CXCL2 (MIP-2 alpha), Chemokine (C-C motif) ligand 1/2/3;CEACAM3, Carcinoembryonic antigen-related cell adhesion molecule 3; PTGS2, prostaglandin-endoperoxide synthase 2; ITGB3, integrin beta 3; LTBP1, latent transforming growth factor beta binding protein 1; EGF, epidermal growth factor; OAS3, 2-;5- oligoadenylate synthetase 3; ELOVL7, ELOVL fatty acid elongase 7; MX1, MC dynamin-like GTPase 1; TNFAIP3, Tumour necrosis factor, alpha-induced protein 3; GREM2, gremlin 2; IL1B, interleukin 1 beta; FCAR, Fc fragment of IgA receptor; MX2, MX dynamin-like GTPase 2.
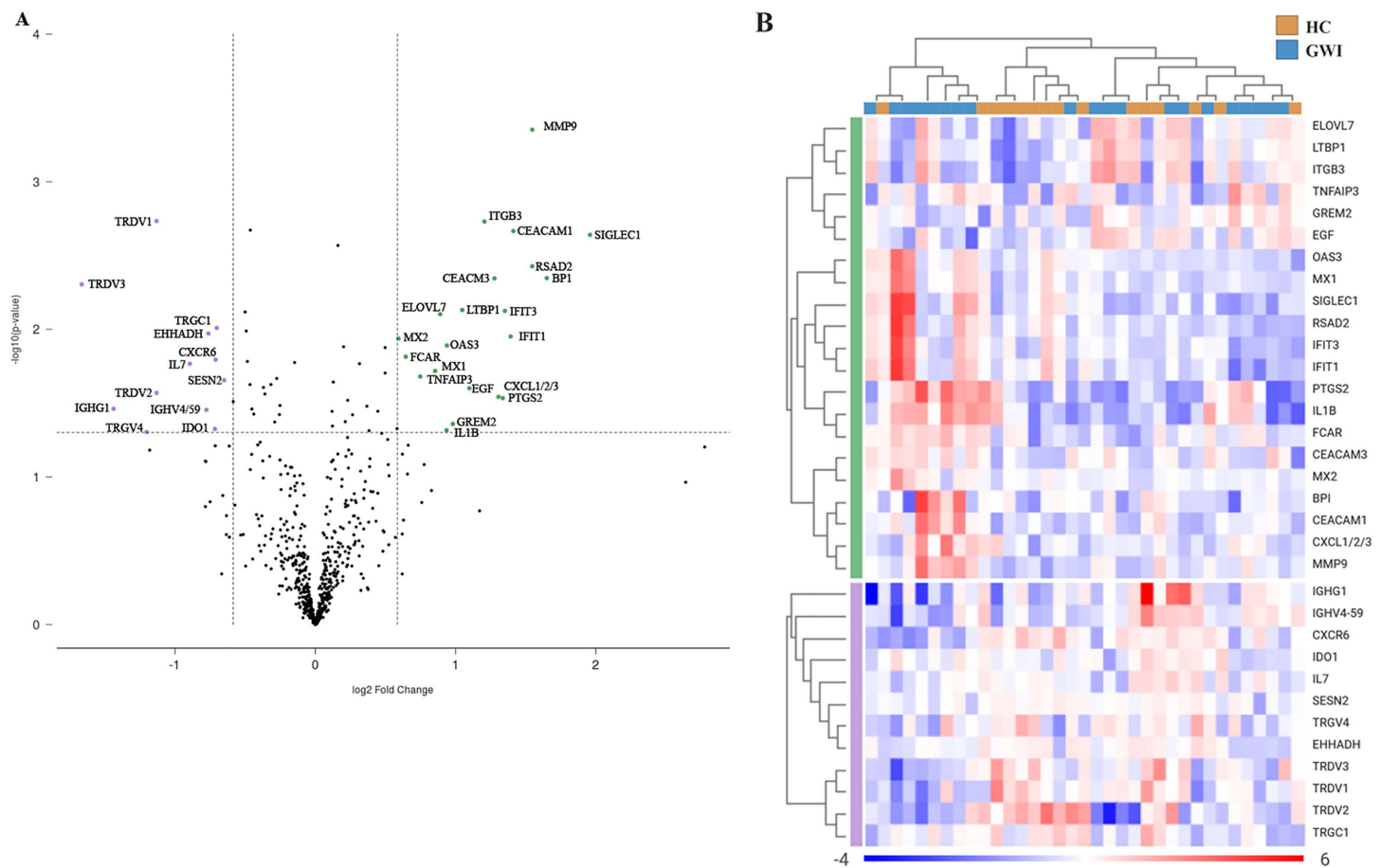
Fig 1. Differentially expressed genes in Australian GWI. (A) volcano plot displaying statistical significance (log10(p-value) on the y-axis, and log2 fold change on the x-axis. Selected genes meeting filter criteria are presented as those down-regulated (≤−1.5) and those upregulated (≥1.5). (B) heatmap of selected genes representing log2 normalised expression values from −4 to 6. Red indicates high levels of expression, while blue indicates low levels of expression. Clusters are organised according to upregulated or downregulated genes by participant cohort. Green indicates upregulated genes, while purple indicates downregulated genes. Figure exported from Rosalind Bio. *Abbreviations: HC, healthy control; GWI, Gulf War Illness; TRDV3, T cell receptor delta variable 3; IGHG1, Immunoglobulin heavy constant gamma 1; TRDV2, T cell receptor delta variable 2; TPSAB1/B2, tryptase alpha/beta 1; TRGV4, T cell receptor gamma variable 4; TRDV1, T cell receptor delta variable 1; IL7, Interleukin 7; IGHV4−59, immunoglobulin heavy variable 4−59; IDO1, indoleamine 2 3-dioxygenase 1; EHHDAH, enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase; CXCR6 chemokine (C-X-C motif) receptor 6; TRGC1, T cell receptor gamma constant 1; SESN2, sestrin 2; SIGLEC, sialic acid binding Ig-like 1; BPI, bactericidal/permeability-increasing protein; RSAD2, radical S-adenosyl methionine domain containing 2; MMP9, Matrix metallopeptidase 9; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; CEACAM1, Carcinoembryonic antigen-related cell adhesion molecule 1; IFIT3, interferon-induced protein with tetratricopeptide repeats 3; CXCL1/2/3, CXCL1, CXCL2 (MIP-2 alpha), Chemokine (C-C motif) ligand 1/2/3;CEACAM3, Carcinoembryonic antigen-related cell adhesion molecule 3; PTGS2, prostaglandin-endoperoxide synthase 2; ITGB3, integrin beta 3; LTBP1, latent transforming growth factor beta binding protein 1; EGF, epidermal growth factor; OAS3, 2-;5- oligoadenylate synthetase 3; ELOVL7, ELOVL fatty acid elongase 7; MX1, MC dynamin-like GTPase 1; TNFAIP3, Tumour necrosis factor, alpha-induced protein 3; GREM2, gremlin 2; IL1B, interleukin 1 beta; FCAR, Fc fragment of IgA receptor; MX2, MX dynamin-like GTPase 2.*

Downregulation of TCRs and impaired T cell function are reported in various pathologies, including cancer, autoimmune diseases and infectious diseases [30]. Similarly, TCR dysregulation may contributed to the changes in T cell subsets and pro-inflammatory responses observed in GWI. Immune profiling and function have been investigated and have reported altered T- and NK lymphocyte profiles and impaired cytotoxic function, with emphasis on elevated T lymphocyte populations of veterans with GWI compared with controls [13,14,31]. Whether the downregulation of TCRs is correlated to a

Table 5. Gene set analysis for genes differentially expression in Australian GWI.

GSA	UES	DES
Type I Interferon	1.7835	1.7386
NK Receptors	1.6857	−0.1878
Fatty Acid Metabolism	1.4838	−1.0185
TNF Signalling	1.3493	0.9285
TCR Signalling	1.319	−0.7643
Hypoxia Response	1.2441	0.9354
IL-7 Signalling	1.2037	−0.5323
PPAR Signalling	1.1884	−1.1031
Chemokine Signalling	1.1768	−0.8164
Other IL Signalling	1.1676	0.7214

Data extracted from Rosalind Bio. Abbreviations: GSA, gene set analysis; UES, undirected enrichment score; DES, directed enrichment score; NK, natural killer; TNF, tumour necrosis factor, TCR, T cell receptor; IL, interleukin; PPAR, peroxisome proliferator-activated receptors.

https://doi.org/10.1371/journal.pone.0338198.t005

potential elevation in T cell populations are unknown. This current investigation did not directly quantify lymphocyte cell numbers and cell profiling according to differentially expressed genes did not significantly differ between GWI and HC cohorts. Instead, future investigations may aim to concurrently analyse gene expression with phenotyping. Notably, treatment with PB, a medication routinely administered as a prophylactic during the Gulf War, can impede T cells through the cholinergic anti-inflammatory pathway (CAP) resulting in the suppression of T cell activity [32]. However, the implications of long-term or excessive PB, as reported by GWV, on T cell activity is unknown. Nevertheless, reduced TCR functioning in GWI is a novel finding and supports the need for further investigations into immune disturbances in Australian GWV.

Upregulated interferon-related genes including *RSAD2*, *IFIT1*, *IFIT3*, *MX1*, *MX2* and *OAS3* suggests chronic activation of antiviral and inflammatory pathways. This is further supported by GSA data whereby type I interferon associated genes returned a positive DES, as seen above. Type I interferon activation suggests persistent immune activation. While elevated interferon signalling may also suggest automimicry in this cohort of GWI, the downregulation of *IGHG1* and *IGHV4–59* suggests reduced humoral immune responses and impaired antibody production, not reminiscent of the presence of autoimmunity which is inconsistent in GWI research [33,34]. Upregulation of other inflammatory and immune activation markers reported in the present manuscript, such as *IL1B*, *PTGS2*, *TNFAIP3*, *CXCL1/2/3* and *FCAR* aligns further with chronic inflammation or innate immune activation [35–38]. In further support of our research, a previous investigation reported elevated levels of IFN-y- in addition to IL-2-producing CD4 + cells and elevated *in vitro* levels of IL-10-producing CD4 cells compared with non-symptomatic GWV [39]. While plasma levels of IL-6 and C-reactive protein (CRP) are also found to be increased in veterans with GWI [40]. Therefore, the present research, in conjunction with the existing literature, supports the role of chronic inflammation in the pathomechanism of GWI.

The downstream implications of the preceding exposures and resulting chronic inflammation are potentially associated with metabolic disturbances reported in GWI. Results of the present investigation suggest metabolic dysfunction evidenced through differential expression of genes including *EGF*, *ITGB3*, *LTBP1*, *GREM2, SESN2*, and *ELOVL7*. For example, the downregulation of *SESN2,* encoding for sestrin 2, suggests mitochondrial deoxyribonucleic acid damage, oxidative stress, and hypoxia reported in human disease, including those that are neurodegenerative [41]. Previous investigations have reported that impaired mitochondrial function is associated with symptom severity in veterans with GWI [42]. A review of research on inflammation and ROS suggests that inflammatory mediators, such as those reported in GWI cohorts, may potentially exacerbate metabolic dysfunction and fatty acid oxidation [43]. Suggesting that inflammation and mitochondrial dynamics are interconnected in disease. Additionally, Bryant *et al.* reported that altered cellular metabolism
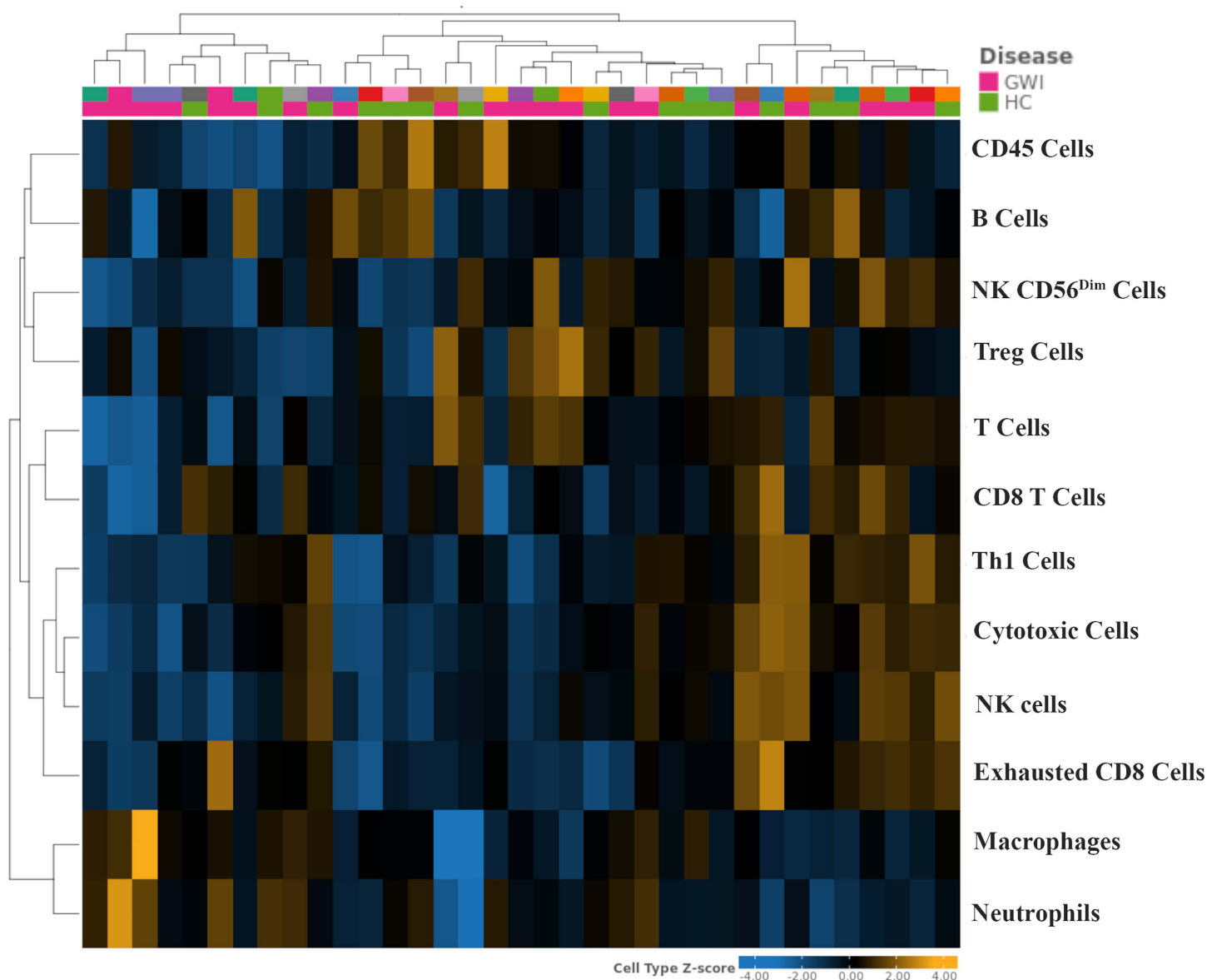
Fig 2. Cell profiles and gene expression. (A) Heatmap extracted from Rosalind Bio. Cell type z-score for cell populations were populated for samples collected from GWI and HC. *Abbreviations: NK, natural killer; Treg, T regulatory; Th, T helper.*

https://doi.org/10.1371/journal.pone.0338198.g002

in a GWI mouse model may promote inflammatory processes in veterans; however, further protein- and functional-level research is required to determine whether this is supported by the present study. The downstream effects of genes including *SESN2* and others listed above on cellular metabolism may indeed exacerbate inflammation aligning with Bryant *et al* [26]. Other research has reported on genetic variants linked with mitochondrial disturbances, such as *BChE* [24]. While transcriptomics research in GWI mice have also reported differential expression of genes important for mitochondrial respiration, oxidative phosphorylation and electron transport chain [44], the top differentially expressed genes are not replicated by the present investigation. Differences in the literature may be linked to the use of a GWI mouse model [26,44] or samples provide by veterans with GWI [42,45], therefore, posing a barrier when interpreting data between models.

Table 6. Top biological functions and pathways in Australian GWI.

| Functions	P-value	Molecules
Childhood-onset SLE	<0.0001	RSAD2,SIGLEC1
ATAD3A-related type I interferonopathy	<0.0001	RSAD2,SIGLEC1
SLE	<0.0001	RSAD2,BPI,MMP9,SIGLEC1
Activation of leukocytes	<0.0001	RSAD2,BPI,MMP9,SIGLEC1
Antineutrophil cytoplasmic antibody-associated vasculitis	<0.0001	RSAD2,MMP9,SIGLEC1
Pathways	**P-value**	**Ratio**
Neutrophil Extracellular Trap Signalling Pathway	0.0023	0.00489
Neutrophil degranulation	0.0031	0.00419
Role of Lipids/Lipid Rafts in the pathogenesis of influenza	0.0045	0.0417
Chaperone Mediate Autophagy Signalling Pathway	0.0055	0.00314
Toll-like Receptor Cascades	0.006	0.0312

Data extracted from IPA. Ratio is calculated as the number of molecules in a given pathway that meets cutoff criteria, divided by the total number of molecules that make up that pathway and that are in the reference set. *Abbreviations: SLE, systemic lupus erythematosus; SIGLEC, sialic acid binding Ig-like 1; BPI, bactericidal/permeability-increasing protein; RSAD2, radical S-adenosyl methionine domain containing 2; MMP9, Matrix metallopeptidase 9.*

Overall, this present research is supported by the literature reporting a decline in metabolic function in veterans with GWI and therefore provides avenues for future research [45–47].

Further, GSA suggested the potential occurrence of impaired fatty acid metabolism in the present GWI cohort. While the BMI of veterans with GWI was significantly higher compared with HC, previous research using a GWI mouse model demonstrated mitochondrial lipid changes in the brains and plasma [48]. This mouse model was exposed to GW agents PB and permethrin, therefore, it can be hypothesised that mitochondrial disturbances reported in this present manuscript are potentially linked to exposures of GWV and the role of fatty acid metabolism disturbances cannot be limited to BMI in this cohort. This is further supported by multivariate analysis undertaken to determine association between BMI and gene expression which found potential associated with the expression of *IGHV4/59* and *CEACAM3* and not genes related to fatty acid metabolism (S2 Table). Nevertheless, further research is warranted to determine the impact of high BMI on the regulation of metabolic genes, as the sample size of this research did not allow data stratification on BMI.

Currently, there is no validated biomarker for GWI for diagnosis or to determine risk susceptibility. The identification of biological markers could help to refine illness definition, better detect, predict or distinguish subgroups of GWI, and ultimately lead to the development of hypothesis-driven and evidence-based treatments to improve health outcomes of veterans. While the occurrence of immune exhaustion was investigated using a targeted genomic panel, typical immune exhaustion markers such as Programmed Death-1 (PD-1), lymphocyte activating gene (LAG), T cell immunoreceptor ITIM domain 3 (TIM-3) and cytotoxic T lymphocyte associated proteins (CTLAs) [49], were not identified as differentially expression. Rather, the results of the current investigation suggest the role of chronic immune activation and inflammation in Australians with GWI. Given the impact of immune responses linked to GWI symptomology, genetic variability that causes persistent inflammatory or immune alterations may be essential for further research into diagnostic tools or targeted pharmacotherapeutic intervention. This present research provides the foundations to facilitate further analysis for potential biomarker identification specific to GWI. This current manuscript further elucidates the role of immunological disturbances in the pathomechanism of GWI. Importantly, the mechanisms involved in the immune disturbances reported in GWI are potentially linked with immunotoxicity caused by exposures during the Gulf War resulting in lymphocyte dysfunction, increased oxidative stress, and dysregulation of immune signalling pathways [5,6,50].
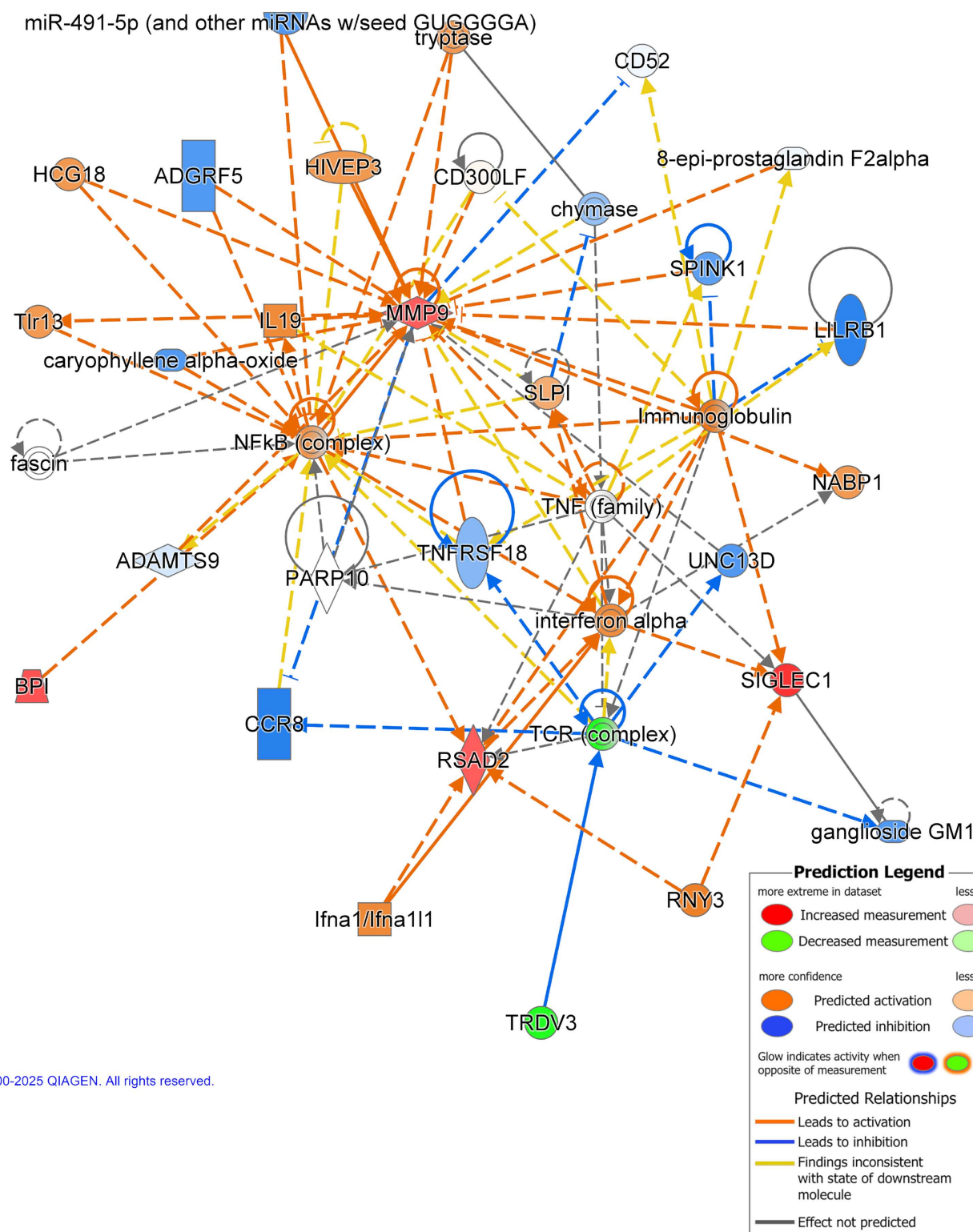
Fig 3. Network analysis in Australian GWI. Gene interaction network map consisting of top filtered differentially expressed genes. Genes are organised according to subcellular space. Network analysis score = 16. *Abbreviations: TRDV3, T Cell Receptor Delta Variable 3; RNY3, RNA, Ro-Associated Y3; SIGLEC1, Sialic Acid Binding Ig Like Lectin 1; NABP1, Nucleic Acid Binding Protein 1; RSAD2, Radical S-Adenosyl Methionine Domain Containing*

2; CCR8, C-C Motif Chemokine Receptor 8; TCR, T Cell Receptor; BPI, Bactericidal/Permeability-Increasing Protein; ADAMTS9, ADAM Metallopepti-dase With Thrombospondin Type 1 Motif 9; PARP10, Poly(ADP-Ribose) Polymerase Family Member 10; TNFRSF18, Tumor Necrosis Factor Receptor Superfamily Member 18; TNF, Tumor Necrosis Factor; SLPI, Secretory Leukocyte Peptidase Inhibitor; NFkB, Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells; IL19, Interleukin 19; ADGRF5, Adhesion G Protein-Coupled Receptor F5; HCG18, HLA Complex Group 18; TIR13, Likely refers to TIR domain-containing protein 13, though this is not a well-characterized molecule; MMP9, Matrix Metallopeptidase 9; SPINK1, Serine Peptidase Inhibitor, Kazal Type 1; LILRB1,Leukocyte Immunoglobulin-Like Receptor Subfamily B Member 1; HIVEP3, Human Immunodeficiency Virus Type I Enhancer Binding Protein 3. Figure constructed using IPA, Qiagen._

This current investigation is not without limitations. The small cohort sizes limit stratification of cohorts according to clinical presentation, age, BMI, and other potential confounding factors. This emphasises the need for further investigations with larger cohorts to differentiate potential immune subtypes and identify biomarkers for stratification. Nevertheless, effect sizes calculated for each differentially expressed gene were found to be moderate to large demonstrating a sufficient sample size to support these findings (S2 Table). With further investigations incorporating larger sample sizes and protein-level validation may also be considered for future research. Given the significant differences in age between the cohorts, a multivariate analysis was performed to determine any potential effect on gene expression. We report that the effect of age on gene expression was non-significant, excluding one gene being *IGHV4/59* (S2 Table). It is important to highlight that while this current investigation raised disease pathways associated with SLE, no participants reported a diagnosis of an autoimmune condition. This current investigation serves as the basis to justify further larger investigations to identify immunological biomarkers in GWI. Further, the Immune Exhaustion panel developed by NanoString biases expression analysis to a small selection of genes. While this technology provides sensitive data, future analysis may consider the validation of gene expression analysis using untargeted RNA expression analysis with quantitative polymerase chain reaction experiments to confirm findings.

Conclusion

This investigation reports immune transcriptome changes in Australian veterans with GWI using NanoString Immune Exhaustion panel. Altered gene expression identified in this study indicates changes to both innate and adaptive immune responses with evidence of metabolic stress, and IL signalling disturbances. The findings of this present research suggest chronic inflammation is a potential mechanism underpinning symptom presentation of GWI. Markers of immune exhaustion were not statistically different in Australian GWI participants. Moreover, these findings contribute to the growing body of literature on the pathomechanism of immunological disturbances in GWI and may facilitate further research in the identification of diagnostic or therapeutic targets.

Supporting information

S1 Table. This file contains pre-processed gene expression data collected prior to statistical or pathway analyses. Information and data was generated using NanoString nCounter Immune Exhaustion Panel and Rosalind Bio. Inside the file contains a full list of genes and probes included in the NanoString nCounter Immune Exhaustion Panel including probe IDs, gene symbols and probe sequences (T1); lists corresponding functional themes of genes incorporated within the panel supporting predetermined information used for gene set and pathway analysis (T2); a summary of panel coverage according to genes within the panel contributing to immune-related pathways or cell types (T3); raw gene expression for housekeeping genes (T4); normalised RNA expression counts subsequent to positive control normalisation and background removal for all genes fulfilling thresholding requirements (T5); raw RNA gene expression counts prior to normalisation or quality control (T6) and; a summary of quality check data including binding density, image quality, limit of detection, and more, for each sample calculated using NanoString nSolver and nCounter Analysis (T7).
(XLSX)

S2 Table. This file contains results from statistical analyses performed and summaries within the results of this manuscript. Inside the file contains lists all normalised gene counts that passed quality checks and thresholding with corresponding samples including base mean, $\log_2$ fold change, unadjusted p-values, adjusted p-values, false discovery rate and significance rank (T1); filtered list of only significant differentially expressed genes (T2); full results pertaining to the gene set analysis performed using Rosalind Bio (T3); standardised effect sizes (Cohen's D) for all statistically significant differentially expressed genes calculated using R package "RNASeqPower" and "effsize"; lists the effect of metadata variables Age and BMI on gene expression profiles across cohorts (GWI or HC) using multivariate analysis performed with SPSS (T5).
(XLSX)

S3 Table. File provides immune cell type abundance scores using gene expression information. The $\log_2$ cell type abundance scores are derived from cell-specific genes using Rosalind Bio. This data was used to generate Figure 2 of the results.
(XLSX)

S4 Table. This file documents functional and pathway interpretation for differentially expressed genes using QIAGEN IPA according to [22]. File contains Diseases and Functions according to differential gene expression including p-values and molecules (T1); lists canonical pathways along with corresponding molecules, direction of expression and pathway activity predictions including p-values, z-score, ratio and molecules (T2); causal network analyses predicting upstream and downstream regulators and networks involving specific gene families including direction of activation or inhibition, p-value, and molecules (T3); lists predicted upstream regulators according to observed gene expression changes with corresponding p-values and molecules (T4); underlying network molecules and key diseases or functions used for Figure 3 included in the present manuscript detailing key molecules (T5).
(XLSX)

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References

1. Blanchard MS, Eisen SA, Alpern R, Karlinsky J, Toomey R, Reda DJ, et al. Chronic multisymptom illness complex in Gulf War I veterans 10 years later. Am J Epidemiol. 2006;163(1):66–75. https://doi.org/10.1093/aje/kwj008 PMID: 16293719

2. Binns J, Bloom F, Bunker J, Crawford F, Golomb B, Klimas N, et al. Gulf War Illness and the Health of Gulf War Veterans: Research Update and Recommendations. 2014.

3. Kelsall HL, McKenzie DP, Sim MR, Leder K, Forbes AB, Dwyer T. Physical, psychological, and functional comorbidities of multisymptom illness in Australian male veterans of the 1991 Gulf War. Am J Epidemiol. 2009;170(8):1048–56. https://doi.org/10.1093/aje/kwp238 PMID: 19762370

4. White RF, Steele L, O'Callaghan JP, Sullivan K, Binns JH, Golomb BA, et al. Recent research on Gulf War illness and other health problems in veterans of the 1991 Gulf War: Effects of toxicant exposures during deployment. Cortex. 2016;74:449–75. https://doi.org/10.1016/j.cortex.2015.08.022 PMID: 26493934

5. Bou Zerdan M, Moussa S, Atoui A, Assi HI. Mechanisms of Immunotoxicity: Stressors and Evaluators. Int J Mol Sci. 2021;22(15):8242. https://doi.org/10.3390/ijms22158242 PMID: 34361007

6. Semwal R, Semwal RB, Lehmann J, Semwal DK. Recent advances in immunotoxicity and its impact on human health: causative agents, effects and existing treatments. Int Immunopharmacol. 2022;108:108859. https://doi.org/10.1016/j.intimp.2022.108859 PMID: 35598397

7. Mitra A, Sarkar M, Chatterjee C. Modulation of Immune Response by Organophosphate Pesticides: Mammals as Potential Model. Proc Zool Soc. 2017;72(1):13–24. https://doi.org/10.1007/s12595-017-0256-5

8. Burzynski HE, Macht VA, Woodruff JL, Crawford JN, Erichsen JM, Piroli GG, et al. Pyridostigmine bromide elicits progressive and chronic impairments in the cholinergic anti-inflammatory pathway in the prefrontal cortex and hippocampus of male rats. Neurobiol Stress. 2022;18:100446. https://doi.org/10.1016/j.ynstr.2022.100446 PMID: 35573808

9. Joyce MR, Holton KF. Neurotoxicity in Gulf War Illness and the potential role of glutamate. Neurotoxicology. 2020;80:60–70. https://doi.org/10.1016/j.neuro.2020.06.008 PMID: 32585289

10. Brooks AW, Sandri BJ, Nixon JP, Nurkiewicz TR, Barach P, Trembley JH, et al. Neuroinflammation and Brain Health Risks in Veterans Exposed to Burn Pit Toxins. IJMS. 2024;25(18):9759. https://doi.org/10.3390/ijms25189759

11. Joshi U, Pearson A, Evans JE, Langlois H, Saltiel N, Ojo J, et al. A permethrin metabolite is associated with adaptive immune responses in Gulf War Illness. Brain Behav Immun. 2019;81:545–59. https://doi.org/10.1016/j.bbi.2019.07.015 PMID: 31325531

12. Shetty GA, Hattiangady B, Upadhya D, Bates A, Attaluri S, Shuai B, et al. Chronic Oxidative Stress, Mitochondrial Dysfunction, Nrf2 Activation and Inflammation in the Hippocampus Accompany Heightened Systemic Inflammation and Oxidative Stress in an Animal Model of Gulf War Illness. Front Mol Neurosci. 2017;10:182. https://doi.org/10.3389/fnmol.2017.00182 PMID: 28659758

13. Whistler T, Fletcher MA, Lonergan W, Zeng X-R, Lin J-M, Laperriere A, et al. Impaired immune function in Gulf War Illness. BMC Med Genomics. 2009;2:12. https://doi.org/10.1186/1755-8794-2-12 PMID: 19265525

14. Vojdani A, Thrasher JD. Cellular and humoral immune abnormalities in Gulf War veterans. Environ Health Perspect. 2004;112(8):840–6. https://doi.org/10.1289/ehp.6881

15. Broderick G, Kreitz A, Fuite J, Fletcher MA, Vernon SD, Klimas N. A pilot study of immune network remodeling under challenge in Gulf War Illness. Brain Behav Immun. 2011;25(2):302–13. https://doi.org/10.1016/j.bbi.2010.10.011 PMID: 20955779

16. Sultana E, Shastry N, Kasarla R, Hardy J, Collado F, Aenlle K, et al. Disentangling the effects of PTSD from Gulf War Illness in male veterans via a systems-wide analysis of immune cell, cytokine, and symptom measures. Military Med Res. 2024;11(1). https://doi.org/10.1186/s40779-023-00505-4

17. Coller JK, Tuke J, Wain TJ, Quinn E, Steele L, Abreu M, et al. Associations of Immune Genetic Variability with Gulf War Illness in 1990–1991 Gulf War Veterans from the Gulf War Illness Consortium (GWIC) Multisite Case-Control Study. Brain Sciences. 2021;11(11):1410. https://doi.org/10.3390/brainsci11111410

18. Fukuda K. Chronic Multisymptom Illness Affecting Air Force Veterans of the Gulf War. JAMA. 1998;280(11):981. https://doi.org/10.1001/jama.280.11.981

19. Reyes M, Nisenbaum R, Hoaglin DC, Unger ER, Emmons C, Randall B, et al. Prevalence and incidence of chronic fatigue syndrome in Wichita, Kansas. Arch Intern Med. 2003;163(13):1530–6. https://doi.org/10.1001/archinte.163.13.1530 PMID: 12860574

20. Panda S, Ravindran B. Isolation of Human PBMCs. Bio-protocol. 2013;3(3). https://doi.org/10.21769/bioprotoc.323

21. Eaton-Fitch N, Rudd P, Er T, Hool L, Herrero L, Marshall-Gradisnik S. Immune exhaustion in ME/CFS and long COVID. JCI Insight. 2024;9(20):e183810. https://doi.org/10.1172/jci.insight.183810 PMID: 39435656

22. Krämer A, Green J, Pollard J Jr, Tugendreich S. Causal analysis approaches in Ingenuity Pathway Analysis. Bioinformatics. 2014;30(4):523–30. https://doi.org/10.1093/bioinformatics/btt703 PMID: 24336805

23. Haley RW, Kramer G, Xiao J, Dever JA, Teiber JF. Evaluation of a Gene-Environment Interaction of PON1 and Low-Level Nerve Agent Exposure with Gulf War Illness: A Prevalence Case-Control Study Drawn from the U.S. Military Health Survey's National Population Sample. Environ Health Perspect. 2022;130(5):57001. https://doi.org/10.1289/EHP9009 PMID: 35543525

24. Golomb BA, Kelley RI, Han JH, Miller B, Bui L. Gulf war illness: a tale of two genomes. BMC Res Notes. 2024;17(1):230. https://doi.org/10.1186/s13104-024-06871-z PMID: 39169443

25. Abdullah L, Nkiliza A, Niedospial D, Aldrich G, Bartenfelder G, Keegan A, et al. Genetic association between the APOE ε4 allele, toxicant exposures and Gulf war illness diagnosis. Environ Health. 2023;22(1):51. https://doi.org/10.1186/s12940-023-01002-w PMID: 37415220

26. Bryant JD, Kodali M, Shuai B, Menissy SS, Graves PJ, Phan TT, et al. Neuroimmune mechanisms of cognitive impairment in a mouse model of Gulf War illness. Brain Behav Immun. 2021;97:204–18. https://doi.org/10.1016/j.bbi.2021.07.015 PMID: 34333111

27. James LM, Christova P, Engdahl BE, Lewis SM, Carpenter AF, Georgopoulos AP. Human Leukocyte Antigen (HLA) and Gulf War Illness (GWI): HLA-DRB1*13:02 Spares Subcortical Atrophy in Gulf War Veterans. EBioMedicine. 2017;26:126–31. https://doi.org/10.1016/j.ebiom.2017.11.005 PMID: 29137891

28. San José E, Borroto A, Niedergang F, Alcover A, Alarcón B. Triggering the TCR complex causes the downregulation of nonengaged receptors by a signal transduction-dependent mechanism. Immunity. 2000;12(2):161–70. https://doi.org/10.1016/s1074-7613(00)80169-7 PMID: 10714682

29. Ross SH, Cantrell DA. Signaling and Function of Interleukin-2 in T Lymphocytes. Annu Rev Immunol. 2018;36:411–33. https://doi.org/10.1146/annurev-immunol-042617-053352 PMID: 29677473

30. Baniyash M. TCR zeta-chain downregulation: curtailing an excessive inflammatory immune response. Nat Rev Immunol. 2004;4(9):675–87. https://doi.org/10.1038/nri1434 PMID: 15343367

31. Zhang Q, Zhou X-D, 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(1):6–13. https://doi.org/10.1128/cdli.6.1.6-13.1999

32. Valdés-Ferrer SI, Crispín JC, Belaunzarán PF, Cantú-Brito CG, Sierra-Madero J, Alcocer-Varela J. Acetylcholine-esterase inhibitor pyridostigmine decreases T cell overactivation in patients infected by HIV. AIDS Res Hum Retroviruses. 2009;25(8):749–55. https://doi.org/10.1089/aid.2008.0257 PMID: 19645607

33. Phillips CJ, Matyas GR, Hansen CJ, Alving CR, Smith TC, Ryan MAK. Antibodies to squalene in US Navy Persian Gulf War veterans with chronic multisymptom illness. Vaccine. 2009;27(29):3921–6. https://doi.org/10.1016/j.vaccine.2009.03.091 PMID: 19379786

34. Asa PB, Cao Y, Garry RF. Antibodies to squalene in Gulf War syndrome. Exp Mol Pathol. 2000;68(1):55–64. https://doi.org/10.1006/exmp.1999.2295 PMID: 10640454

35. Lopez-Castejon G, Brough D. Understanding the mechanism of IL-1β secretion. Cytokine & Growth Factor Reviews. 2011;22(4):189–95. https://doi.org/10.1016/j.cytogfr.2011.10.001

36. Mendiola AS, Cardona AE. The IL-1β phenomena in neuroinflammatory diseases. J Neural Transm. 2017;125(5):781–95. https://doi.org/10.1007/s00702-017-1732-9

37. Ren K, Torres R. Role of interleukin-1beta during pain and inflammation. Brain Res Rev. 2009;60(1):57–64. https://doi.org/10.1016/j.brainres-rev.2008.12.020 PMID: 19166877

38. Martín-Vázquez E, Cobo-Vuilleumier N, López-Noriega L, Lorenzo PI, Gauthier BR. The PTGS2/COX2-PGE2 signaling cascade in inflammation: Pro or anti? A case study with type 1 diabetes mellitus. Int J Biol Sci. 2023;19(13):4157–65. https://doi.org/10.7150/ijbs.86492 PMID: 37705740

39. 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(1):66–73. https://doi.org/10.1023/B:JOCI.0000018065.64685.82 PMID: 14997036

40. Butterick TA, Trembley JH, Hocum Stone LL, Muller CJ, Rudquist RR, Bach RR. Gulf War Illness-associated increases in blood levels of interleukin 6 and C-reactive protein: biomarker evidence of inflammation. BMC Res Notes. 2019;12(1):816. https://doi.org/10.1186/s13104-019-4855-2 PMID: 31852524

41. Chen Y, Huang T, Yu Z, Yu Q, Wang Y, Hu J, et al. The functions and roles of sestrins in regulating human diseases. Cell Mol Biol Lett. 2022;27(1):2. https://doi.org/10.1186/s11658-021-00302-8 PMID: 34979914

42. Golomb BA, Sanchez Baez R, Schilling JM, Dhanani M, Fannon MJ, Berg BK, et al. Mitochondrial impairment but not peripheral inflammation predicts greater Gulf War illness severity. Sci Rep. 2023;13(1):10739. https://doi.org/10.1038/s41598-023-35896-w PMID: 37438460

43. Nesci S, Spagnoletta A, Oppedisano F. Inflammation, Mitochondria and Natural Compounds Together in the Circle of Trust. Int J Mol Sci. 2023;24(7):6106. https://doi.org/10.3390/ijms24076106 PMID: 37047080

44. Xu F, Ashbrook DG, Gao J, Starlard-Davenport A, Zhao W, Miller DB, et al. Genome-wide transcriptome architecture in a mouse model of Gulf War Illness. Brain Behav Immun. 2020;89:209–23. https://doi.org/10.1016/j.bbi.2020.06.018 PMID: 32574576

45. Naviaux RK, Naviaux JC, Li K, Wang L, Monk JM, Bright AT, et al. Metabolic features of Gulf War illness. PLoS One. 2019;14(7):e0219531. https://doi.org/10.1371/journal.pone.0219531 PMID: 31348786

46. Raju RP, Terry AV. Dysregulation of cellular energetics in Gulf War Illness. Toxicology. 2021;461:152894. https://doi.org/10.1016/j.tox.2021.152894 PMID: 34389359

47. Hokama Y, Empey-Campora C, Hara C, Higa N, Siu N, Lau R, et al. Acute phase phospholipids related to the cardiolipin of mitochondria in the sera of patients with chronic fatigue syndrome (CFS), chronic Ciguatera fish poisoning (CCFP), and other diseases attributed to chemicals, Gulf War, and marine toxins. J Clin Lab Anal. 2008;22(2):99–105. https://doi.org/10.1002/jcla.20217 PMID: 18348309

48. Abdullah L, Evans JE, Joshi U, Crynen G, Reed J, Mouzon B, et al. Translational potential of long-term decreases in mitochondrial lipids in a mouse model of Gulf War Illness. Toxicology. 2016;372:22–33. https://doi.org/10.1016/j.tox.2016.10.012 PMID: 27931520

49. Blank CU, Haining WN, Held W, Hogan PG, Kallies A, Lugli E, et al. Defining "T cell exhaustion". Nat Rev Immunol. 2019;19(11):665–74. https://doi.org/10.1038/s41577-019-0221-9 PMID: 31570879

50. Trageser KJ, Sebastian-Valverde M, Naughton SX, Pasinetti GM. The Innate Immune System and Inflammatory Priming: Potential Mechanistic Factors in Mood Disorders and Gulf War Illness. Front Psychiatry. 2020;11:704. https://doi.org/10.3389/fpsyt.2020.00704 PMID: 32848904