AWARD NUMBER: W81XWH-13-2-0072

TITLE: Brain Immune Interactions as the Basis of Gulf War Illness: Gulf War Illness Consortium (GWIC)

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CONTRACTING ORGANIZATION: Boston University Medical Campus, Boston , MA

REPORT DATE: October 2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2020	Annual	30Sep2019-29Sep2020

4. TITLE AND SUBTITLE

Brain Immune Interactions as the Basis of Gulf War Illness: Gulf War Illness Consortium (GWIC)

5a. CONTRACT NUMBER
W81XWH-13-2-0072

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The primary function of the Gulf War Illness (GWIC) consortium is to identify the pathobiological mechanisms of Gulf War Illness. The ultimate goal is to discover and characterize biomarkers of Gulf War illness and then identify targeted treatment strategies. The GWIC allows for the development of multidisciplinary collaborations targeting suspected brain- immune signaling alterations in GWI. The GWIC consortium central hypothesis identifies chronic neuroinflammation as an end result of initial glial activation and subsequent priming of glial responses that cause a chronic activation loop of stronger and longer proinflammatory signaling effects between the immune system and the brain. The GWIC includes both clinical (human) and preclinical (animal and cell) studies and researchers in the 10 funded sub-studies. These studies are incorporating sufficient overlap of scientific content area to inform each other in a bench-to-bedside-to-bench approach. Results to date from the preclinical (animal) studies suggest a strong neuroinflammatory component to the illness model and provide important leads for treatment development approaches in the animal model before translation to the clinic. Clinical study recruitment is ongoing and has shown correlations between proinflammatory cytokine markers and behavioral and neuroimaging outcomes. The final larger sample size will continue to make these inter-relationships clearer.

15. SUBJECT TERMS
Gulf War Illness, consortium, CNS, innate immunity, cytokines, MRI neuroimaging, pesticides, DFP, sarin

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	140	**19b. TELEPHONE NUMBER** *(include area code)*

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1. INTRODUCTION:

The primary function of the Gulf War Illness (GWIC) consortium is to identify the pathobiological mechanisms of Gulf War Illness. The ultimate goal is to discover and characterize biomarkers of Gulf War illness and then identify targeted treatment strategies. The GWIC allows for the development of multidisciplinary collaborations targeting suspected brain-immune signaling alterations in GWI. The GWIC consortium central hypothesis identifies chronic neuroinflammation as an end result of initial glial activation and subsequent priming of glial responses that cause a chronic activation loop of stronger and longer proinflammatory signaling effects between the immune system and the brain. The GWIC includes both clinical (human) and preclinical (animal and cell) studies and researchers in the 10 funded sub-studies.

2. KEYWORDS:

Gulf War Illness, consortium, CNS, innate immunity, cytokines, MRI neuroimaging, cognitive deficits, pesticides, DFP, sarin, CORT, genetics, objective biomarkers, treatment development

3. OVERALL PROJECT SUMMARY:

INTRODUCTION

Background. Twenty-nine years after the 1991 Gulf War, 30% of the nearly 700,000 U.S. troops who served in the war still suffer from the debilitating symptomatic illness known as Gulf War Illness (GWI) (White et al., 2016; RAC, 2008, 2014, IOM, 2010). A growing body of evidence indicates that GWI is associated with diverse central nervous system (CNS) and immune alterations, but the specific pathobiological processes driving GWI symptoms have not been clearly elucidated (Zhang et al., 1999; Sullivan et al, 2003; Heaton et al., 2007; Toomey et al., 2009; Whistler et al., 2009; Broderick et al., 2011; Chao et al., 2011; Sullivan et al., 2013; White et al., 2016; Abou-Donia et al., 2017; Janulewicz et al., 2017; Jeffrey et al., 2019; Belgrad et al., 2019; Joshi et al., 2019). Animal studies indicate that a chronic CNS inflammatory state can develop in response to an insult—chemical injury, infection, or physical trauma (including mild traumatic brain injury)—that mobilizes CNS defense systems via activation of glia, the brain's primary immune response cells, and release of chemical messengers that precipitate a complex of "sickness behavior symptoms" identified by measures of impaired memory and learning, increased pain sensitivity, and persistent fatigue, a symptom complex similar to that of GWI (Rathbone et al., 2015; Banks & Lein, 2012; Watkins et al., 2007; 2009; Zhang et al., 2010). Recent studies have also demonstrated CNS inflammatory effects of GW-related exposures and additional immune and cellular processes that plausibly explain the mechanisms contributing to the full spectrum of GWI symptoms (Miller and O'Callaghan 2019; Belgrad et al., 2019; Joshi et al., 2019; Janulewicz et al., 2019; Januelwicz et al., 2018; Abou-Donia et al., 2017; Koo et al., 2017; Rao et al., 2017; Qiang et al., 2017; Locker et al., 2017; Emmerich et al., 2017; Abdullah et al., 2016; O'Callaghan et al., 2016; O'Callaghan et al., 2015; Milligan et al., 2009; Rivest et al., 2009; Spradling et al., 2011).

Consortium Management and Expertise. This multidisciplinary collaboration brings together established GWI researchers, and leading experts in brain-immune processes associated with neurotoxicology and neuroinflammation, damage to white matter and axonal transport, immunology, and immunogenetics. This team has designed a body of interrelated studies linked together by a cohesive model of 'brain-immune interactions' as the basis for GWI. The consortium is led by Dr. Kimberly Sullivan, at Boston University (BU), whose extensive background in GWI research includes contributions in identifying effects of Gulf War exposures on brain structure and function (Sullivan et al., 2003; Sullivan et al., 2013; Yee et al., 2015; Yee et al., 2017; Janulewicz et al., 2017; Janulewicz et al., 2018; Sullivan et al., 2018; Jeffrey et al., 2019). BU serves as the Coordinating Center for the Gulf War Illness Consortium (GWIC) and provides the Administrative and Data Management Cores (figure 1). The consortium also includes a Preclinical Core, consisting of experts at five sites who are working collaboratively to characterize the persistent neurological and immune effects of GW exposures at the physiological, tissue, and cellular levels. This is done

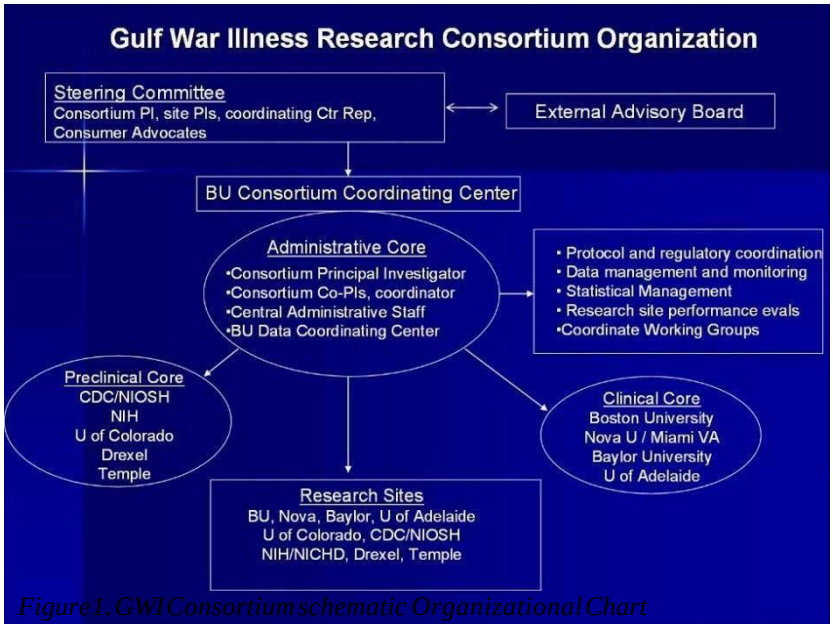


Figure 1. GWI Consortium schematic Organizational Chart

in parallel with human studies conducted by the Clinical Core at three recruitment sites (and two additional laboratory sites) to characterize the specific profile of brain, immune, and genetic measures that distinguish veterans with GWI from healthy controls. The GWIC Steering Committee and External Advisory Committee monitors research progress and findings, and advises on research modifications and follow-up.

Objective. The primary objective of the Boston GWI consortium is to provide a cohesive understanding of the pathobiological mechanisms for the symptoms of GWI in order to provide a rational and efficient basis for identifying beneficial treatments and diagnostic markers.

Research Plan. The consortium is undertaking a coordinated series of clinical and preclinical studies aimed at providing a comprehensive understanding of the pathobiology of GWI. This includes clinical case-control studies conducted in parallel at 3 subject recruitment sites—Boston, Miami, and Houston—that include a total of 300 Gulf War veterans. Clinical assessments include a) advanced neuroimaging protocols (MRI, DTI, fMRI, PET) that assess brain volumetrics, white matter integrity, and CNS inflammatory indicators, b) neuropsychological assessment of cognitive function, c) blood levels of cytokines and other immune signaling molecules, d) genetic expression of immune markers, e) pilot assessment of cerebrospinal fluid levels (CSF) of cytokines and neurotransmitters (in subgroup of Boston cohort), f) immunogenetic markers of innate immune responsivity, f) longitudinal assessment of brain-immune measures. Parallel preclinical studies are evaluating persistent effects of GW neurotoxicants *in vitro* and in rodent models of GWI. Preclinical studies are evaluating cellular effects of GW neurotoxicants on a) axonal transport, b) glial cytokine production, c) neurotransmitter signaling, d) myelination, and e) oligodendrocyte proliferation. Animal studies are determining the effects of GW exposures on: a) priming and maintaining glial activation, differentiating effects on astrocytes vs. microglia, b) glial activation in relation to development of learning impairment and chronic pain sensitivity, c) brain and blood levels of proinflammatory cytokines, and d) genetic expression of immune and inflammatory markers in brain and blood. Findings from clinical and preclinical studies are being compared and used to identify specific brain-immune pathways that can be targeted for intervention by a variety of glial modulating and other currently available treatments. Treatment compounds are being tested in animal models to determine their effectiveness for resolving or ameliorating the pathobiological processes associated with GWI. Figure 1 represents the hypothesized mechanisms for GWI that are being tested by this planned series of preclinical and clinical experiments.

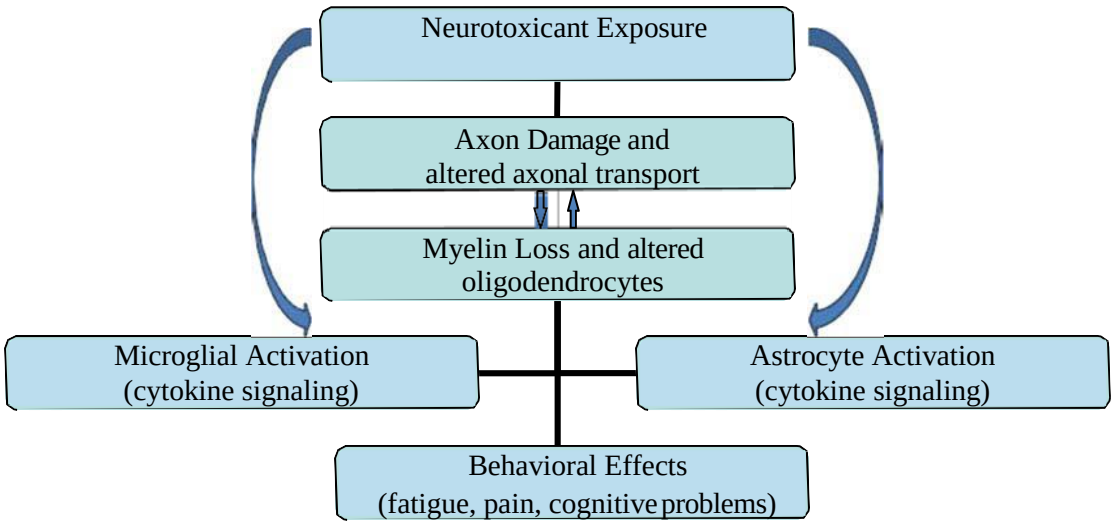
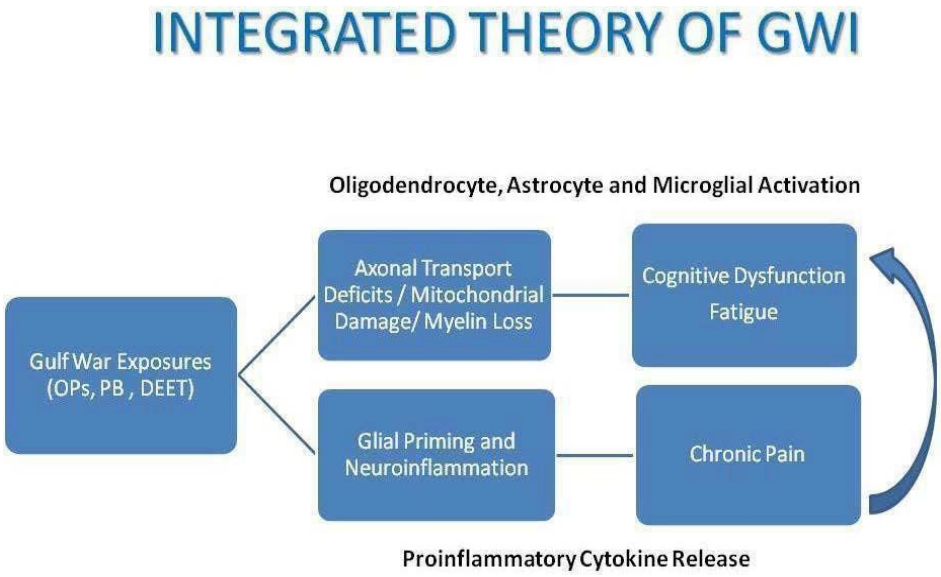


Figure 2. Schematic Representation of Hypothesized GWI Mechanisms

The GWI consortium central hypothesis identifies chronic neuroinflammation as an end result of initial glial *activation* and subsequent *priming* of glial responses that cause a chronic activation loop of stronger and longer proinflammatory effects between the immune system and the brain. Figure 2 below represents the integrated theory of GWI being tested in the consortium studies.



The overall aims of this integrated multidisciplinary consortium scientific focus are to (1) To identify validated markers of GW illness by using state of the art neuroimaging, behavioral, genetic and blood markers of neuroinflammatory activation in both clinical and preclinical models that will elucidate targeted and validated treatment strategies (2) To create a Neuroinflammation Risk Profile for GWI (3) To identify viable mechanistic treatments based on identified pathophysiological pathways of GWI that have been validated in preclinical treatment models.

BODY

The approved statement of work for the entire study period is below:

STATEMENT OF WORK

Table 1. Brain-Immune Interactions as the Basis of Gulf War Illness: Gulf War Illness Consortium

Task 1. Obtain necessary authorization prior to initiation of human subjects' and animal studies research (months 1-8)
1a. Attend pre-award meeting with CDMRP GWIRP program staff
1b. Obtain final Institutional Review Board (IRB) approval for clinical research sites at Boston University School of Public Health (BUSPH), Baylor University and Miami VA/Nova University for protocols and advertisements
1c. Obtain final DOD Human subjects Research Protections Office (HRPO) approvals
1d. Obtain data use agreement from Hines VA for stored blood sample study
1e. Obtain final protocol approval by the respective Institutional Animal Care and Use Committees (IACUC) approval for the preclinical animal research sites at Center f Disease Control/NIOSH, National Institutes of Health, Drexel University, Temp University and University of Colorado
1f. Complete hiring of necessary staff and ensure all mandatory IRB and IACUC research related trainings are completed by all staff members
Task 2. Preparation for consortium clinical studies (months 1-9)
2a. BUSPH Data Coordinating Center (DCC) will create website, data collection forms, specimen tracking system and databases for the entire consortium including all preclinical and clinical sites.
2b. Develop manuals for the neuropsychological testing protocol, imaging protocols, specimen collection protocols and recruitment.
2c. Train researchers and staff on protocols and quality control measures for the clinical and preclinical studies.
2d. Obtain stored blood samples from Hines VA study and send to Miami VA for analysis.
Task 3. Preparation for consortium preclinical studies (months 9 - 24)
3a. Prepare rat dosing models at CDC and distribute to other sites at NIH, Drexel, Temple and U-Colorado for planned studies of axonal transport, myelin integrity and learning and pain assessments.
3b. Develop co-cultures of rodent oligodendrocytes in cell culture chambers for electrical stimulation of axons and development of myelination in vitro at NIH.
Task 4. Perform preclinical cell and animal studies (months 9-42)
4a. Assess for axonal transport integrity in rodent and cell models exposed to either GW-relevant neurotoxicants or cytokines (Drexel - 30 Sprague Dawley rats, Temple - 27 Sprague Dawley rats).
4b. Assess for myelin integrity in rodent and cell models exposed to either GW-relevant neurotoxicants or cytokines (NIH – 624 NIH/S mice and 208 rats).
4c. Assess whether persistent priming of neuroinflammation occurs chronically with GW-relevant neurotoxicants and intermittent corticosterone exposure to model the chronic nature of GWI (CDC – 100 C57BL/6 mice).

4d. Assess the relative contributions of astrocytes and microglia in rodent GWI neuroinflammatory models in order to identify which glial markers will provide the best candidate "drugable" targets (CDC 40 C57BL/6 mice; 40 ALDH1L1 mice; 40 B6.129-Cx3CR1 mice).
4e. Assess the relationship between behavioral testing of learning and memory and enhanced pain, in rodent GWI neuroinflammatory models by assessing hippocampal functioning with a fear conditioning task (U-Colorado – 120 rats).
4f. Compare central and peripheral markers of neuroinflammation in brain tissue and blood samples from GWI neuroinflammatory rodent models (CDC – 60 rats, Nova).
4g. Compare the effectiveness of several relevant preclinical treatments for GWI in cell and animal studies, including inflammatory glial activation modulators, antioxidants, and neuroprotective peptides (Drexel, Temple, CDC, U-Colorado)(20 animals per treatment).
Task 5. Screening, recruitment and assessment of Gulf War veterans from three sites (months 9-42)
5a. Obtain informed consent from potentially eligible GW veterans
5b. Assess subjects by obtaining demographics, medical history, self-report questionnaires, neuropsychological testing, brain imaging and blood draw and saliva samples.
5c. Upload neuroimaging data to BUSPH for post-processing of MR images and for data analysis.
5d. Score neuropsychological tests and upload summary data to DCC for entry, cleaning and analyses.
5e. Send blood and saliva samples to Nova University for analysis of cytokine and chemokine panels and cortisol measurements.
5f. Send additional saliva samples to University of Adelaide for genetic polymorphism analysis
5g. Conduct preliminary analyses of clinical data
Task 6. Recruitment and assessment for Boston CSF and PET studies (months 24-42)
6a. Perform lumbar punctures to obtain cerebrospinal fluid markers of neuroinflammation in 50 GW veterans.
6b. Perform positron emission tomography (PET) scanning with novel EAAT2 ligand in partnership with RIO pharmaceuticals in 15 GW veterans.
6c. Perform FDG-PET scan imaging with 30 GW veterans after a computerized CPT cognitive challenge task.
Task. 7. Interim Analyses, Grant Submission, and Annual Reporting (Months 18-42)
7a. Data entry of all questionnaires, evaluations and quality control measures will be ongoing
7b. Interim Statistical analyses of data obtained from cognitive evaluations, blood markers, neuroimaging and questionnaire data will be performed periodically.
7c. Grant submissions to relevant funding agencies for further collaborative studies based on initial results and preliminary data targeted toward treatment strategies will be ongoing.
7d. Annual reports of progress will be written.
Task 8. Final analysis and Report Writing (months 42-48)
8a. Statistical analyses comparing brain MRI volumetrics, cognitive functioning, health symptom report and cytokine/chemokine markers in veterans with and without GWI

	8b. Statistical analyses of correlations between clinical and preclinical neuroinflammatory markers of GWI models
	8c. Perform longitudinal assessments of imaging, cognitive, health symptom and cytokine functioning in veterans with and without GWI
	8d. Perform validation analysis studies of identified biomarkers of GWI using an unrelated sample of stored blood and cognitive health symptom data from a prior CSP study.
	8e. Write final study report
	8f. Present findings at scientific meetings
	8g. Prepare manuscripts for submission
	8h. Write grant proposals based on consortium findings and identified treatment avenues for GWI.

The statement of work for year 6 is inclusive of Tasks 1-8 above. The statement of work for year 6 primarily describes the completion of the initial 10 sub-studies. In addition, in year 6, the plan was to have cell and animal studies all underway or completed, reporting final results and publishing manuscripts. The plan was also to continue with subject recruitment for the clinical studies and to recruit 145 study participants for the study protocol including cognitive evaluations, interviews, neuroimaging and specimen collection. Progress toward completing each task is listed below and due to some delays in finishing the ten studies, a no-cost extension was requested and approved for a seventh years to complete the GWIC studies.

TASK 1. OBTAIN NECESSARY AUTHORIZATION PRIOR TO INITIATION OF HUMAN SUBJECTS' AND ANIMAL STUDIES RESEARCH (MONTHS 1-8)

Task 1a. Attend pre-award meeting with CDMRP GWIRP program staff
Due to delays in funding the consortium as a result of the government shutdown, the pre-award meeting was held in February 2013 and was considered a post-award meeting. The meeting included an overview of study hypotheses and plans as well as a review of the consortium administrative and core center structure. The Consortium PI, Dr. Sullivan and other steering committee members were present at the meeting in addition to CDMRP commanders, grants officer's representative (GOR) and administrative staff. Required External Advisory Board (EAB) meetings have also begun to meet with the first meeting being held in September 2014. Subsequent EAB meetings were held in April 2015, October 2015, May 2016, November 2016; May 2017 and November 2017; May 2018. The EAB provided helpful suggestions and comments for study progress and discussions for future meetings that have occurred semi-annually during the consortium funding period.

Task 1b. Obtain final Institutional Review Board (IRB) approval for clinical research sites at Boston University School of Public Health (BUSPH), Baylor University and Miami VA/Nova University for protocols and advertisements
IRB and HRPO approvals have been submitted and approved for all three clinical sites at Miami VA/NOVA University, Boston University (BU) and Baylor College of Medicine and renewed as required. University of Adelaide received exempt status from their local IRB.

Task 1c. Obtain final DOD Human subjects Research Protections Office (HRPO) approvals
HRPO submissions have been submitted and approved for all three sites at Miami VA/NOVA University, Boston University and Baylor Medical College.

Task 1d. Obtain data use agreement from Hines VA for stored blood sample study

Study Description: Analysis of stored blood samples from a previous large multi-site VA study of GWI (CSP#458) will be used to examine the relationships between proinflammatory cytokine markers and the behavioral symptoms associated with GWI, such as impaired memory and pain. This study will validate clinical results from the main study in an unrelated GW veteran cohort. We will access de-identified stored bloods and study data from the CSP #458. Subject data will come from GW veterans who were all

originally enrolled in The National Health Survey of Gulf War Era Veterans and Their Families and who were determined to have CMI (n= 327). We will sample 100 veterans, divided equally into 50 with Khamisiyah exposure and 50 without. These two groups will be matched on CMI illness severity (mild-moderate vs. severe). The control sample will include 100 DV without CMI. The CMI group and controls will be matched to the extent possible on demographic variables.

DUA document has been finalized between BU and Hines VA and signed by the BU attorney. Final revised and IRB approved versions of the DUAs from Boston and Miami were sent to VA Central Office and have now been approved and signed. Blood samples have now been sent to Dr. Klimas at the Miami VA for cytokine analysis. Dr. Klimas will perform the cytokine analyses and send the results to Hines VA. Hines VA will conduct the statistical analyses and complete this validation study in the next quarter.

Task 1e. Obtain final protocol approval by the respective Institutional Animal Care and Use Committees (IACUC) approval for the preclinical animal research sites at Center for Disease Control/NIOSH, National Institutes of Health, Drexel University, Temple University and University of Colorado

All local IACUC approvals have been obtained from CDC, NIH, Temple and University of Colorado. BU offsite IACUC approvals have been obtained for all animal study sites. ACURO final approvals have also been obtained for all pre-clinical sites and renewals are submitted for approval as they are required for 3-year re-writes. Most animal studies are now completed with the exception of the CDC site where treatment studies remain ongoing.

Task 1f. Complete hiring of necessary staff and ensure all mandatory IRB and IACUC research related trainings are completed by all staff members

Hiring of local post-docs and research assistants has been ongoing for each site. BUSPH hired and trained a new consortium project coordinator and research assistant, and Dr. Steele hired and trained a research assistant for the clinical studies at Baylor to complete subject recruitment there. All current staff have completed IRB and IACUC trainings necessary for their work with animal and human studies. The Miami site hired and trained an additional research assistant to complete recruitment for the study at their site. Only the BU site continues to recruit for control participants at this time.

TASK 2. PREPARATION FOR CONSORTIUM CLINICAL STUDIES

The consortium coordinating center and Administrative Core at Boston University has led many monthly web and in-person meetings during the past year to oversee and interact with the clinical study sites. A significant amount of time and effort was devoted to maintain all required study and test administration materials and training new staff members. Table 2 lists these planning meetings. Smaller working group meetings were also held during the past year to plan for particular consortium topic areas. The Working Groups are described in Table 3. Since subject recruitment has been largely completed, considerable time has been spent with training and quality control assurance meetings of clinical staff to ensure consistent inter-rater reliability and to reduce any administration drift from standard testing and scoring procedures. Baylor College of Medicine and Miami VA sites have now completed recruitment and clinical appointments at their sites. Boston added new clinical staff to their team to complete subject recruitment. With these new changes, training on test administration and quality control measures have continued for all three sites.

Table 2. GWIC Monthly Planning and EAB Meetings for 2018

Date	Type of Meeting	Discussion Items
11-06-2019	Monthly Web-meeting	Pre-clinical and clinical study updates
12-04-2019	Monthly Web-meeting	Pre-clinical and clinical study updates
01-08-2020	Monthly Web-meeting	Pre-clinical and clinical study updates
02-05-2020	Monthly Web-meeting	Pre-clinical and clinical study updates

03-04-2020	Monthly Web-meeting	Pre-clinical and clinical study updates
04-01-2020	Monthly Web-meeting	Pre-clinical and clinical study updates
05-06-2020	Monthly Web-meeting	Pre-clinical and clinical study updates
06-03-2020	GWIC in-person meeting	Pre-clinical and clinical study updates
07-01-2020	Monthly Web-meeting	Pre-clinical and clinical study updates
08-05-2020	Monthly Web-meeting	Pre-clinical and clinical study updates
09-02-2020	Monthly Web-meeting	Pre-clinical and clinical study updates
10-07-2020	Monthly Web-meeting	Pre-clinical and clinical study updates

Table 3. Consortium Working Groups

Working Group	Tasks	Members
Data Management Service Group	Assist with QC issues, data cleaning, data management and sharing, website management.	Joe Palmisano, DCC Consortium PI, co-PIs
Statistics Service Group	Perform analyses and provides statistical planning and advice for study investigators and research site PIs.	Timothy Heeren, Joe Palmisano, Consortium PI/co- PIs
Translational Working Group	Forum for Intellectual property and material (IP) issues, translation of results into papers, abstracts, new grant submissions and how clinical and preclinical results can inform each other.	Michael Pratt – BU Tech Transfer office Consortium PI, co-PIs Research site PIs, RIO
Behavioral Studies Working Group	Plan imaging protocols and provide quality control for multiple imaging sites. Plan behavioral testing protocols and coordinate preclinical and clinical studies for comparability.	Drs. Sullivan, Killiany, Krengel, Toomey, Steele, Klimas, Coller, Hutchinson, Maier, Watkins
Histopathology Working Group	Plan tissue studies of proinflammatory, glial, axonal transport and mitochondrial markers in similarly dosed animal and cell models.	Drs. Baas, O'Callaghan, Fields, Maier, Watkins
Immune Genetics Working Group	Plan and implement studies assessing brain-immune interactions involving glia and proinflammatory cytokines/chemokines through genetic SNPs and mRNA and miRNA protein studies.	Drs. Coller, Hutchinson, Klimas, Steele, Sullivan, Watkins, Maier
Gulf War Veterans Advisory working Group	Update fellow GW veterans about GWIC research efforts and results, assist with recruitment efforts by making fellow vets aware of GWIC studies.	Denise Nichols, Frances Perez Wilhite, Lynn Santosuosso, Tim Demers, Christine Tron, Jim Arrocho

Task 2a. BUSPH Data Coordinating Center (DCC) will create website, data collection forms, specimen tracking system and databases for the entire consortium including all preclinical and clinical sites.

Consortium website (http://sites.bu.edu/gwic/) and other social media pages are finalized and approval was obtained from each institution to use their logos on the site. They are updated regularly and are a primary source of subject recruitment. Electronic data collection forms using REDCap software and CATI recruitment software were finalized and in use for subject screening and data collection. The study is utilizing Frontier Science's LDMS specimen tracking system for shipping samples to collaborating sites and for biorepository tracking. Training for

the specimen tracking system has also been completed by all necessary staff. A refresher REDCap training was completed for all clinical staff and is in use as subjects are recruited and complete online questionnaires and screened for eligibility through the CATI system.

Task 2b. Develop manuals for the neuropsychological testing protocol, imaging protocols, specimen collection protocols and recruitment.

All cognitive administration and scoring manuals, specimen collection protocols have been finalized. All clinical staff has been trained to ensure proper quality control measures are in place for the clinical studies.
This has been followed up by videotaping practice testing to ensure tester drift is not occurring and bi-monthly Zoom meetings were conducted with testing staff as subject recruitment was underway to answer any questions or discuss problems with test administration/scoring issues. This has proven helpful to ensure consistent test administration and scoring at all study sites until completion at the Houston and Miami sites earlier this year.

Amid the Covid-19 pandemic, the BU site has gotten IRB approval to conduct neuropsychological testing via Zoom to continue testing participants. The neuropsychological manual was updated to ensure all cognitive/motor functioning skills are still being tested while it is conducted remotely. Participants will be coming to the BU campus for specimen collection only.

Task 2c. Train researchers and staff on protocols and quality control measures for the clinical and preclinical studies

Training for researchers and clinical staff was completed at in-person meeting in Boston in August 2014 and continued to be monitored as described above. Working groups have finalized training procedures and protocols for cognitive, neuroimaging and laboratory procedures that are currently being used. As new staff members are added, they are trained appropriately.

Task 2d. Obtain stored blood samples from Hines VA study and send to Miami VA for analysis.

Dr. Toomey has obtained local Boston University exempt IRB status for this project. Dr. Klimas has obtained local IRB approval from Miami VA IRB for the study. The DUA document that was finalized between BU and the VA and signed by the BU attorney was put on a newer DUA template by Hines VA staff and the new version was reviewed and signed. The Miami VA IRB has approved the DUA and sent it to the Hines VA. Hines VA reviewed and approved the DUA. Final revised and IRB approved versions of the DUAs from Boston and Miami were sent to VA Central Office for review in mid-June and have received final signature approval in December 2019. Blood samples have now be sent to Dr. Klimas at the Miami VA for analyses and processing. Dr. Klimas will perform the cytokine testing and send the results to the Hines VA. Hines VA will conduct the statistical analyses and report the results back to GWIC for comparison with GWIC results and write-up for publication.

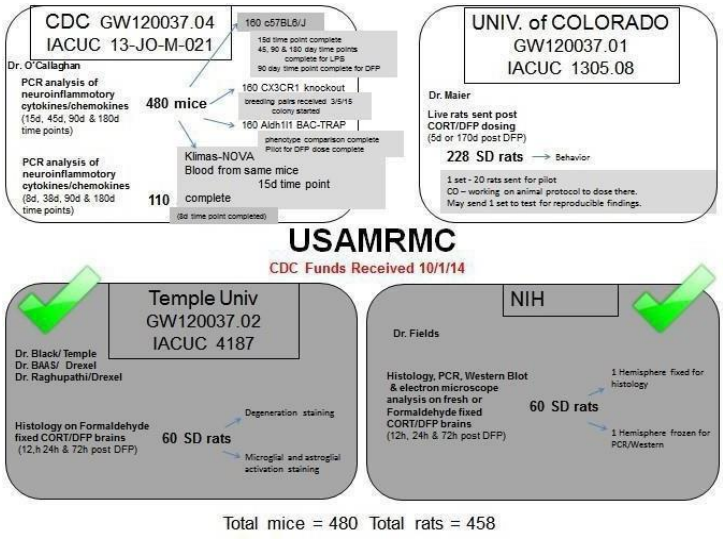
We finalized all paperwork for transferring funds to Hines VA and transferred those funds. We finalized the algorithm for Gulf War Illness using the available data to most closely represent the Kansas definition. We identified the sample and were able to match sub-samples on all our planned for criteria. The final sample of 200 met criteria for a modified Kansas definition and a modified CDC definition of Gulf War Illness. We sent the list and documentation to Boston MAVERIC. Boston MAVERIC mailed the samples to Miami this month and they arrived safely. The next step is for Dr. Klimas and her team to analyze the samples and send the results back to the Hines VA for analysis and comparison with GWIC results. We hope to finalize this study in the next quarter.

TASK 3. PREPARATION FOR CONSORTIUM PRECLINICAL STUDIES (MONTHS 9 - 24)

Monthly web meeting and working group meetings were ongoing during the past year to prepare for the planned preclinical treatment and new pilot studies and to coordinate overlap of the studies and to ensure that

the same neurotoxicant dosing and exposure model of GWI were used in the pilot studies. The CDC site was tasked with comparing the mouse and rat models of GWI to ensure comparability for planned studies and to distribute dosed animals and animal tissue to the preclinical sites which they have successfully done.

Figure 3. Distribution schematic of animals from CDC



This has resulted in several published preclinical and translational papers (Lacagnina et al., 2020; Michalovicz et al., 2020; Belgrad et al., 2019; Michalowicz et al., 2019; Joshi et al., 2019; Janulewicz et al., 2019; Seth et al., 2019; Kimono et al., 2019; Kelly et al., 2018; O'Callaghan et al., 2017; Koo et al., 2017; Rao et al., 2017; Qiang et al., 2017; Locker et al., 2017; Emmerich et al., 2017; Abdullah et al., 2016; O'Callaghan et al., 2016; Fields et al., 2017). Dr. O'Callaghan has traveled to UCSF to assist with the PET animal pilot dosing study with Dr. Gerdes at RIO pharmaceuticals where the pilot study has now been performed and the results will be presented in the next quarter.

Task 3a. Prepare rat dosing models at CDC and distribute to other sites at NIH, Drexel, Temple and U-Colorado for planned studies of axonal transport, myelin integrity and learning and pain assessments.

Dr. O'Callaghan at the CDC site prepared and validated rat dosing models based on his initial mouse GWI dosing models from prior DOD funded studies using chronic daily corticosterone (CORT) and 1 dosage of the sarin-surrogate DFP (O'Callaghan et al., 2015).

CDC sacrificed 20 adult male rats [control group (n= 5), CORT only exposed group (n= 5), acute DFP exposed group (n= 5), and chronic CORT and DFP group (n= 5)]. Animals in CORT groups were exposed to 200mg/L in their drinking water for 4 days followed by a single s.c. injection of saline (CORT group) or 1.5mg/kg DFP (CORT and DFP). Rats in the control and DFP groups were given standard tap water for 4 days followed by a single s.c. injection of saline (Control) or 1.5mg/kg DFP (DFP). Rats were perfused and brains were preserved in 10% formalin brains. Perfused brains were then sent out to Dr. Killiany and Boston University collaborators for brains to be structurally imaged. These results were published in Koo et al., 2017. Additional perfused brains from the 5-week time point representing current GWI veteran time points were shipped to Dr. Koo at Boston University and were brain imaged. Results will follow shortly.

CDC has exposed rats to the same conditions described above and has shipped flash frozen and formalin preserved brains of rats sacrificed at 7, 14 and 21 days post-DFP exposure to NIH collaborators. Animals in the CORT groups were exposed to 200mg/L in their drinking water for 4 days followed by a single s.c. injection of saline (CORT group) or 1.5mg/kg DFP (CORT and DFP). Rats in the control and DFP groups were given standard tap water for 4 days followed by a single s.c. injection of saline (Control) or 1.5mg/kg DFP (DFP). The 7, 14, and 21 day time points have allowed for investigation of neuronal changes or degradation at further time points post condition exposure and showed important changes over time. These results were published by NIH investigators in Belgrad et al., 2019 and Fields et al., 2017. A review paper on Acetylcholinesterase inhibitors impact on GWI was recently published by Michalowicz et al., 2020.

Task 3b. Develop co-cultures of rodent oligodendrocytes in cell culture chambers for electrical stimulation of axons and development of myelination in vitro at NIH.

Dr. Fields and Dutta made cell cultures from embryonic mice and rats for studies of myelination and the co- cultures were developed in the cell culture chambers for the ongoing oligodendrocyte and myelin studies.

Cholinergic neurons were myelinated in co-culture with oligodendrocytes, providing a framework to study the effects of GW agents on axonal myelination by oligodendrocytes. Techniques were developed to produce purified motor neuron cultures. OPCs were combined with these neuronal cultures in multicompartment chambers for electrical stimulation of axons and treatment with pharmacological agents.

GW agents primarily disrupt cholinergic neurotransmission (Fields et al., 2017). Like neurons, oligodendrocytes express several muscarinic receptors, types 1-5, and our preliminary research shows that oligodendroglia can respond to cholinergic stimulation. We acquired and housed muscarinic receptor 1-5 KO mice, from which oligodendrocytes have been isolated and cultured with GW agents, so as to definitively delineate the role of these agents on oligodendrocyte biology and development.

NIH investigators also received brain tissue from CDC to perform histopathology studies of myelin from exposed animals. The brains from rats exposed to neurotoxicants by Dr. O'Callaghan's lab were analyzed for changes in myelin and other proteins by western blot. This included 60 samples (brains), 12 treatment conditions, 3 time points, (12, 24 and 72 hr) for the proteins olig 2, MBP, and GAP-43. The results show that myelin proteins are affected, but the result is surprising. Rather than decreasing, as would be expected with myelin damage, we find an increase in myelin basic protein (MBP) levels increase significantly 72 hrs after treatment. This result, however, shows that myelinating glia are being affected by the treatments modeling exposures that patients with GWI are likely to have experienced. This is an important finding with respect to the research on white matter damage found in GWI. We suspected that the increase in MBP at this time point may reflect an adaptive response to the toxicants in an attempt to recover from white matter injury. Histological analysis of the same brains have allow us to investigate this. We performed microtome sectioning of these brains and immunocytochemical staining to assess this further. These results have been published in Belgrad et al., 2019.

Interestingly, the only treatment condition where MBP levels changed was in the CORT+DFP group. Neither DFP nor CORT alone caused a change. This result is consistent with other results that Dr. O'Callaghan has reported at CDC and in a recent rat brain imaging publication (Michlowicz et al., 2019; Koo et al., 2017; O'Callaghan et al., 2015).

Dr. Fields then planned longer-term treatments to study effects on myelin. In the results of these longer-term analyses, we saw that increase in MBP levels persisted for the CORT+DFP condition in the longer time-points in Dr. O'Callaghan's rats, as compared to controls. Moreover, unlike in earlier time- points (72 hours and less), MBP levels seem to increase modestly with CORT alone and DFP alone treatments too at 7 days and 21 days post-exposure. We are trying to find a biological basis for why this would be so. Additional study has shown that this increase in MBP is not due to myelination of new axonal sprouts as assessed by expression of GAP43, a marker of axonal sprouting and there is no evidence of astrogliosis in the animal model of GWI, as assessed by expression of GFAP. There is an increase in cell proliferation 21 days post treatment however it is not clear why this is happening.

Returning to the earlier aims identifying the cholinergic receptors on oligodendrocytes by using calcium imaging. This work shows a rich array of cholinergic receptors are active in these cells and thus they would be affected by exposure to nerve agents. A review article about this phenomena was recently published (Fields et al., 2017). The main conclusion was that DFP (50 uM) significantly disrupts 1 uM ACh-treatment mediated OPC intracellular calcium kinetics, response frequency, and amplitude of response.

An important second component of Dr. O'Callaghan's in vivo model of GWI is prior exposure to corticosterone.

Corticosterone has complex cellular and systematic effects, which make it difficult at this point to fully understand its mechanism of action in the GWI model. Dr. Fields therefore began testing oligodendrocytes in cell culture and found that these cells do respond to corticosterone treatment by undergoing a sharp rise in intracellular calcium. He found complex interactions between corticosterone and cholinergic receptor activation, which can be additive, synergistic, or antagonistic to the rise in intracellular calcium caused by cholinergic receptor activation. This and the prior MBP and immunocytochemistry findings have now been published in Glia by Belgrad et al., 2019.

Task 4. Perform preclinical cell and animal studies (months 9-42) Specific progress to date is

listed below for each of the sub-studies.

4a. Assess for axonal transport integrity in rodent and cell models exposed to either GW-relevant neurotoxicants or cytokines (Drexel, Temple).

Studies to assess axonal transport and microtubule integrity in vitro and in vivo have been progressing as expected. Our research team continues to explore the effects of GW neurotoxins and cytokines on axonal transport and neuronal function and we have now shifted from the animal studies to the human induced pluripotent stem cell (hiPSC) and organoid (mini-brain) studies.

Items completed for the axonal transport and microtubule integrity group include:

1) Dr. Liang (Oscar) Qiang used neurons from human stem cell lines and from those differentiated from GW veteran-derived induced pluripotent stem cells as part of a now completed New Investigator proposal to Dr. Peter Baas in collaboration with Dr. Sullivan (GW140086).

2) Gulf War neurotoxicants such as organophosphate pesticides and sarin gas have been shown to alter microtubule dynamics, axonal transport, and mitochondrial health, and these deficits are exacerbated by pretreatment with cortisol to mimic the stress of the battlefield. The funded iPSC work was designed to assess axonal transport and microtubule dysfunction in GW relevant exposures including DFP and cortisol (human equivalent of CORT) so that translation from animal to human studies can be compared for biomarkers and treatment development. The hypothesis is that exposure of neurons and/or neuroinflammatory cells to GW toxins caused long-lasting axonal transport/microtubule defects in neurons, and that these defects lead to a loss of microtubule mass, a change in the proportions of stable and labile microtubule mass, and/or flaws in the lattice of the microtubule that lead to abnormalities in how molecular motor proteins and other microtubule-related proteins interact with the microtubule and move down the axon. Dr. Qiang differentiated human induced pluripotent stem cells (hiPSCs) from GWIC veterans into mature neurons, and then exposed to one of three experimental conditions: vehicle control, Cortisol + the sarin gas analog diisopropylfluorophosphate (DFP), or Cortisol+DFP + Monastrol, an inhibitor of the molecular motor protein kinesin-5. Inhibition of kinesin-5 has been shown to increase microtubule mobility and improve the vitality of axons.

Dr. Qiang's lab is currently investigating the effects of the GWI treatment regimen of DFP+cortisol on hiPSC-derived glutamatergic neurons. They have shown that this treatment reduces MT stability, MT dynamics, axonal transport of mitochondria. Although these changes are important characteristics of the cellular effects of DFP+cortisol treatment, it is important to have a functional readout of how these changes alter higher level processes, and the gold standard functional assay in neuroscience is to examine electrical activity. They will use multi-electrode arrays from MultiChannel Systems, in which hiPSC-derived neural progenitor cells are differentiated directly on the electrodes on the array, to record local field potentials of spontaneous and stimulated electrical activity. They will examine relevant parameters such as firing rate, burst frequency, and network synchrony to assess changes due to DFP+cortisol treatment. A preliminary experiment on our hiPSC-derived glutamatergic neurons indicates that exposure to DFP+cortisol increases neuronal activity, and that hiPSC-derived neurons from veterans with GWI show increased activity compared to control veterans,

suggesting a possible role of hyperexcitability in GWI. This is meaningful because aberrant glutamatergic activity is implicated in numerous cognitive disorders, and GW-relevant organophosphates have been shown to enhance glutamatergic neurotransmission. More importantly, changes in electrical activity can link back to some of the cognitive symptoms of GWI, such as altered information processing speeds and memory deficit.

4b. Assess for myelin integrity in rodent and cell models exposed to either GW-relevant neurotoxicants or cytokines (NIH). –

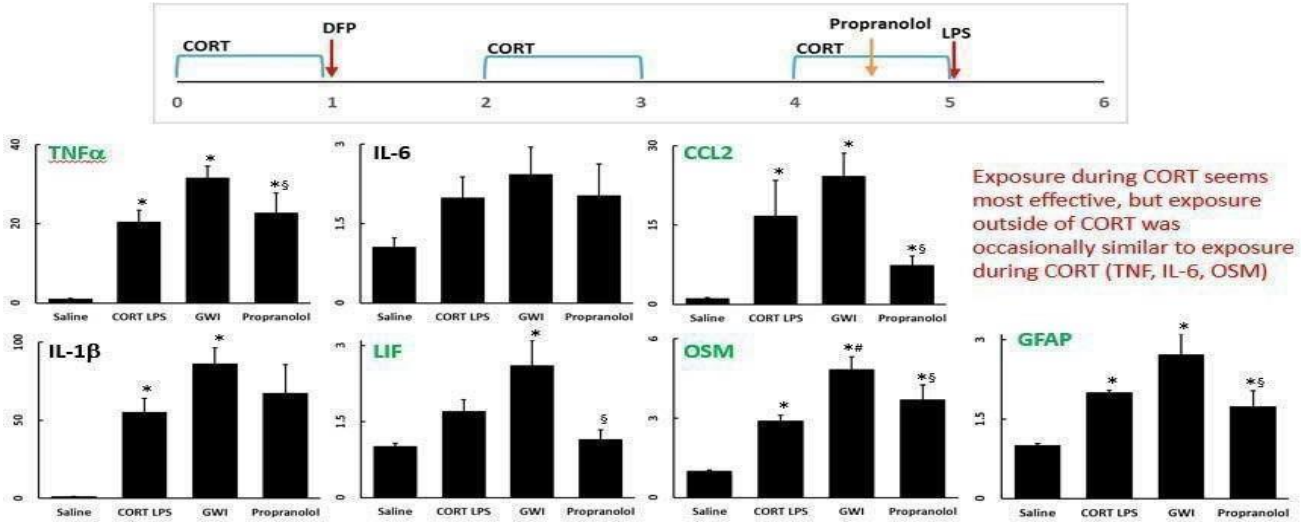
The main conclusions from the NIH work that is now completed can be summed up as: GW toxicants (DFP and CORT) primarily affect:

1. Calcium signaling in oligodendrocytes, in-vitro
2. Oligodendrocyte biology, especially their survival and proliferation, in-vitro

    1. We have characterized these findings in the O'Callaghan rat model of GWI and published two manuscripts (Fields et al., 2017; Belgrad et al., 2019). Although the O'Callaghan model shows no correlation between cholinergic signaling and chronic neuroinflammation in GWI animal models, the cholinergic signaling does seem to affect myelination as described in the abstract below and recent publications (Belgrad et al., 2019; Fields et al., 2017). These are two distinct and perhaps not mutually exclusive phenomena. Neuroinflammation and myelination changes in GWI can and likely do work through different mechanisms. However, OP induced neuronal and myelin damage caused by increased calcium signaling through cholinergic and/or glutamatergic neurotransmitters can lead to 'danger signals' to TLR4 receptors on microglia to illicit chronic neuroinflammatory cytokine signaling. This seems to validate a main GWIC hypothesis regarding at least one mechanism for chronic neuroinflammatory signaling in GWI; that neural debris in the extracellular spaces can and likely does illicit microglial activation and chronic neuroinflammatory signaling.

Task 4c. Assess whether persistent priming of neuroinflammation occurs chronically with GW-relevant neurotoxicants and intermittent corticosterone exposure to model the chronic nature of GWI (CDC –100 C57BL/6 mice).

a. CDC has completed an initial study of propranolol treatment in the 5 week GWI paradigm. Mice received CORT (200 mg/L) in the drinking water for 7 days followed by a single i.p. injection of DFP (4 mg/kg). Mice then received CORT every other week for 4 additional weeks and an LPS inflammatory challenge (0.5 mg/kg, s.c.) on the last day of the experiment. Mice were sacrificed 6 hours post-LPS exposure and assessed for inflammatory cytokine expression in the cortex.



For the treatment schedule, mice received a single, i.p. injection of propranolol (20 mg/kg) during week 4 (day 24) or 5 (day 31) either outside or during CORT exposure, respectively. Overall, propranolol treatment during CORT exposure significantly reduced the expression of the inflammatory cytokines/chemokines: TNFα, CCL2, LIF, and OSM, as well as the astrocyte marker GFAP. This indicates the potential for propranolol to be an effective treatment for the neuroinflammation associated with GWI. Evaluation of cytokine expression when propranolol was given outside of CORT exposure was slightly less effective, only reducing TNFα, IL-6 and OSM expression.

Previously we have shown acute exposure to the initiating event producing GWI pathobiology (CORT and DFP) did not produce significant glial changes in morphology or neurodegeneration (O'Callaghan et al., 2015). Here, we have used that same initiating event with a systemic inflammatory challenge at 5 weeks with interesting new results.

The astrocyte response to the 5-week GWI phenotype has been previously described. Briefly, the combination of CORT and DFP was able to create a pathology in which a subsequent, systemic low dose LPS challenge was able to produce astrocyte hypertrophy at 24 hours after exposure. Control and challenged GWI phenotype treated astrocytes at high magnification highlight the morphological differences in the astrocytes under these conditions. These results appear to be similar to other animal GWI models showing delayed effects on glia after neurotoxicant exposure (Zakirova et al., 2015; Ojo et al., 2014). Results also could be compatible with blood-brain barrier permeability as suggested by reported increased CNS autoantibodies in GW veterans (Abou Donia et al., 2017; Abou Donia et al., 2020).

A protocol for combined CORT/DFP and mild TBI exposure in rats has been submitted to the CDC NIOSH IACUC for approval for the mTBI pilot project and this study will begin in November 2020.

Task 4d. Assess the relative contributions of astrocytes and microglia in rodent GWI neuroinflammatory models in order to identify which glial markers will provide the best candidate "drugable" targets (CDC 40 C57BL/6 mice; 40 ALDH1L1 mice; 40 B6.129- Cx3CR1 mice).

- ALDH1L1 BAC-TRAP mice have been used for initial studies with the CDC GWI exposure protocol, and we see that DFP exposure produces a similar phenotype in these animals to that seen in C57 exposed animals. The TRAP procedure was then used to isolate actively translating mRNA from astrocytes (ALDH1L1-containing cells) at 6 and 72 hours after DFP exposure with and without CORT. Preliminary data supports the use of this model to understand the enrichment of GWI-relevant molecular signatures and signaling pathways in astrocytes over total tissue expression (mixed cell population), as 110 and 211 significantly altered genes in cortical astrocytes were found to be expressed 10-and 5-fold over total cortex, respectively. Initial functional analysis of the set of 211 genes indicates that these genes are largely involved in immune signaling and show particular enrichment for cytokine and complement factor signaling as well as cancer pathways. This paper has now been published online by Michalowicz et al., 2019 at https://www.ncbi.nlm.nih.gov/pubmed/31222732. This suggests that treatments targeting astrocytes and microglia may be appropriate for GWI treatment development. *This paper was nominated for the Charles Shepard CDC best paper of the year award.*
- CDC has performed a preliminary experiment exposing CX3CR1 KO mice to a short term GWI paradigm. These animals were exposed to 200 mg/L CORT in the drinking water for 7 days followed by a single injection of DFP at 4 mg/kg, i.p. Two days later, the mice were exposed to a single, s.c. injection of 0.5 mg/kg LPS to elicit an inflammatory response. Elimination of CX3CR1prevented the priming of CORT DFP exposures on the subsequent LPS. This suggests that microglial responses are crucial for CORT priming of neuroinflammation and supports a neuroimmune mechanism for GWI.

RIO PET IMAGING PILOT STUDY

- Dr. John Gerdes from RIO pharmaceuticals and Dr. Henry Van Brocklin from UCSF also submitted a proposal to assess the EAAT2 PET ligand in the CDC animal model that was previously approved by the EAB. This study got IACUC and ACURO approval and procedures have been completed. This study will have preliminary results to report regarding astrocyte activation in the rat brain following GW-relevant toxicant exposure on PET imaging in the next quarter. Dr. O'Callaghan travelled to UCSF to assist with

training of animal dosing in November to help initiate and oversee the EAAT2 PET studies. The dosing and imaging phase of the study has been completed. Rats post-mortem brain collecting is also completed.

In Sum: The Aim 1 rat dosing, PET-CT and MR scanning and also the acquisition of post-mortem brain tissues have been completed as previously reported. PET-MR-CT imaging data, in addition to blood profiles, have been processed, comparative statistical plots have been prepared across the three rat groups over time, such that the imaging data is now ready for evaluation against the Aim 2 post mortem EAAT2 and GFAP regional cerebral RNA and protein levels over time.

The Aim 2 rat post-mortem brains have been catalogued, were sent during January to the collaborative laboratory of expert Dr. Rita Sattler of the Barrow Neurological Institute, where analysis of regional cerebral RNA and protein densities of target EAAT2 and marker GFAP were initiated. These analyses were halted during the first half of March based on institutional response to the COVID19 pandemic. Based on the COVID19 pandemic, particularly in Arizona, we are waiting for our Aim2 post-mortem data. When that data comes forward, the Aim 3 data correlation of PET imaging outcomes vs. post-mortems findings will be made, and a final report will be fashioned and sent forward.

For Aim 3, once the Aim 2 post mortem brain data is in hand from Dr. Sattler's lab it will be correlated to the Aim 1 EAAT2 PET imaging data. As of now, we anticipate that this pilot will conclude during the next quarter.

We anticipate that a full pilot project report with all data sets and analyses will be reported shortly.

4e. Assess the relationship between behavioral testing of learning and memory and enhanced pain, in rodent GWI neuroinflammatory models by assessing hippocampal functioning with a fear conditioning task (U-Colorado- 120 rats).

- Drs. Maier and Watkins have completed the two pilot studies for treatment of memory functioning and for treatment of pain with two IL10 based treatments that have been previously reported. These results are listed in the next section under treatments.
- Dr. Grace has now published a manuscript on the GWI animal pain and cytokine model in Lacagnina et al. 2020 available at https://pubmed.ncbi.nlm.nih.gov/33127584/.

4f. Compare central and peripheral markers of neuroinflammation in brain tissue and blood samples from 60 rodent GWI neuroinflammatory models (CDC, Nova). -

CDC sent mouse serum samples to NOVA Southeastern University for cytokine analysis following acute exposure to DFP, as well as following the 3-week and 3-month GWI phenotype paradigms. With the longer exposure periods, both brain and serum samples showed a significant inflammatory priming in the CORT DFP exposed samples that are subsequently challenged with LPS. These results will be submitted for publication in a translational paper indicating similarities with the clinical study results.

4g. Compare the effectiveness of several relevant preclinical treatments for GWI in cell and animal studies, including inflammatory glial activation modulators, antioxidants, and neuroprotective peptides (Drexel, Temple, CDC, U-Colorado).-

These important treatment experiments are now well underway and will provide results in the coming months.

1) As previously mentioned, preparations and initial results for studies using propranolol in chronically symptomatic/exposed animal models have been completed by Dr. O'Callaghan's lab at CDC.

2) Drexel investigators have assessed the role of tubacin on stabilizing microtubules and thus reducing acetylated or unstable microtubules. Using tubacin, an FDA-approved HDAC6 inhibitor, changes after DFP exposure, with or without CORT pretreatment, were stabilized and restored to control levels. Human neurons were also treated with DFP + cortisol and the total acetylation ratio was similar in human cells as with rat neurons. This indicates a potential clinical translational therapeutic strategy for GWI. This manuscript was published in Traffic by Rao et al., in May 2017. Additional HDAC6 treatments that are FDA approved were submitted in a n o w f u n d

e d grant to CDMRP for further high- throughput treatment study with GWIC investigators to further this line of treatment development.

3) CDC ran their Tubacin experiment, AhR modulators and luteolin to study their effectiveness in reducing neuroinflammation. Dr. O'Callaghan is working on subsequent animal protocol approvals.

4) Initial dosing studies for the treatments luteolin, HDAC6 inhibitor: tubastatin, and AhR inhibitors: galangin and alpha-naphthoflavone have been completed and were used to determine an appropriate dose for testing in the 5 week GWI paradigm model that are now ongoing.

5) Dr. Linda Watkins at U-Colorado submitted a pilot study to assess a novel pain treatment in rat GWI animal models called by XT150, which is an interleukin-10 based therapy nearing Investigational New Drug status. It has shown great promise in other animal models including in dogs and horses with chronic pain. This pilot study is now completed with results showing that it increased the pain threshold in rats exposed to the GWI animal model. This suggests IL10 therapies should be further explored in animal and clinical studies. Dr. Peter Grace is following up on this work with a funded grant to further assess these outcomes and treatment avenues with initial results published in Lacagnina et al., 2020.

6) On the clinical side, Drs. Klimas, Sullivan, Krengel and other GWI investigators submitted a grant for a GWI Clinical Trials Consortium where several new Phase I/II treatment trials are planned using the current infrastructure of both the Boston and Miami GWI consortia. This grant was awarded to the group and is gearing up for the first remote studies during the pandemic. Thus, treatment development is occurring at the preclinical and clinical sites simultaneously. Importantly GWICTIC phase I and II trials will target GWIC identified TNF-alpha and microglial neuroinflammatory pathways.

TASK 5. SCREENING, RECRUITMENT AND ASSESSMENT OF GULF WAR VETERANS FROM THREE SITES (MONTHS 9-42)

Participant recruitment and screening is ongoing at only Boston University. Miami and Houston has completed recruitment and are no longer screening participants. Currently 633 participants have been screened and 266 have been assessed at the Boston, Miami and Houston. The Boston site currently has nineteen additional participants in the process of being scheduled now that the pandemic restrictions have been lifted.

Task 5a. Obtain informed consent from potentially eligible GW veterans

Recruitment has been ongoing this quarter and 660 total subjects have contacted GWIC coordinators through print advertising, free newsletters to VSOs or social media outlets. From these contacts, 400 were found eligible to participate in the studies. Those not screened were largely not GW veterans. Of this group, 280 have been scheduled and 266 (224 case, 42 controls) have completed the study protocols at Boston, Miami or Houston sites. Miami and Texas site has completed recruitment. Boston site has completed case recruitment of GWI. Recruitment is ongoing at the Boston site to recruit only GW controls. We are working with WeHealth, a media company to assist in connecting the GWIC research to Healthy Gulf War veterans who may be recruited to participate in the study now that our COVID-19 pandemic restrictions on subject recruitment have now been lifted.

	Total	Boston	Miami	Texas
Number of Subjects Contacted	663	385	79	199
Number of Subjects Screened	636 (95.9%)	369 (95.8%)	79 (100.0%)	188 (94.5%)
Number of Subjects Eligible	403 (63.4%)	217 (58.8%)	69 (87.3%)	117 (62.2%)
Number of Subjects with Appointments Made	282 (70.0%)	144 (66.4%)	64 (92.8%)	74 (63.2%)
Number of Subjects Assessed	266 (94.3%)	143 (99.3%)	50 (78.1%)	73 (98.6%)

	Total		Boston		Miami		Texas	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
Number of Subjects Screened	575	61	333	36	70	9	172	16
Number of Subjects Eligible	350	53	185	32	60	9	105	12
Number of Subjects Assessed	224	42	117	26	42	8	65	8

Task 5b. Assess subjects by obtaining demographics, medical history, self-report questionnaires, neuropsychological testing, brain imaging and blood draw and saliva

As planned, cognitive assessment data are being analyzed with neuroimaging data to assess for brain-behavior relationships in GWI. Demographic outcomes suggest a fairly diverse cohort of veterans in terms of race and gender which is helpful with current gene-exposure and SNP analyses. The most recent results were presented at the June 2018 EAB meeting. Updated results are presented in Table 2 below and show significant differences between groups for exposures during the war (chem/bio weapons, wore pesticide treated uniforms and history of mTBI during or post-GW) when compared by students t-tests or chi-square analyses. Interaction analyses comparing chem/bio exposures and m TBI were also significantly different between the groups (p=.007) when compared by MANOVA (see Figure 1). This indicates that there is significant evidence of increased risk of GWI for those with both chemical weapons (CBW) and m TBI exposure. Also, risk of other medical conditions was compared in the mTBI + CBW group when compared with the nonexposed group. These results were recently published in Janulewicz et al., 2018. This paper was nominated for best paper. Specifically, the risk of having other medical conditions was between 4 and 22 times higher in the mTBI + CBW exposed group compared with the non-exposed group. Kansas GWI criteria domains also showed significantly higher symptom do main scores in the mTBI x CBW exposed group for all symptom domains. The findings also correspond and expand upon findings from the Ft. Devens cohort published by Drs. Krengel, Sullivan, and Janulewicz (Yee et al., 2015; Yee et al., 2017).

Table 1. GWIC Subject Demographics

Gulf War Veteran Participants

	Total group	GWI cases	Controls	P-value
Age	52.6 + 5.9	52.2 + 5.7	54.2 + 6.8	0.0470
Gender				0.1856
Male	225 (84.3)	185 (83.0)	40 (90.9)	
Female	42 (15.7)	37 (17.0)	4 (9.1)	
Race				0.9528
Black/African American	33 (12.7)	28 (13.0)	5 (11.4)	
White/Caucasian	206 (79.5)	170 (79.1)	36 (81.8)	
Asian/Pacific Islander	1 (0.4)	1 (0.5)	0 (0)	
Other/Multiracial	19 (7.3)	16 (7.4)	3 (6.8)	
Years of Education	15.0 + 2.0	15.0 + 2.0	15.0 + 2.0	0.6707
Branch of Service				0.4260
Army	171 (66.0)	139 (64.7)	32 (72.7)	
Navy	35 (13.5)	28 (13.0)	7 (15.9)	
Air Force	18 (6.9)	16 (7.4)	2 (4.5)	
Marines	35 (13.5)	32 (14.9)	3 (6.8)	

Figure 1. Chi-square analyses of mTBI rates in GWI cases and controls pre, during and post Gulf War (Janulewicz et al., 2018)

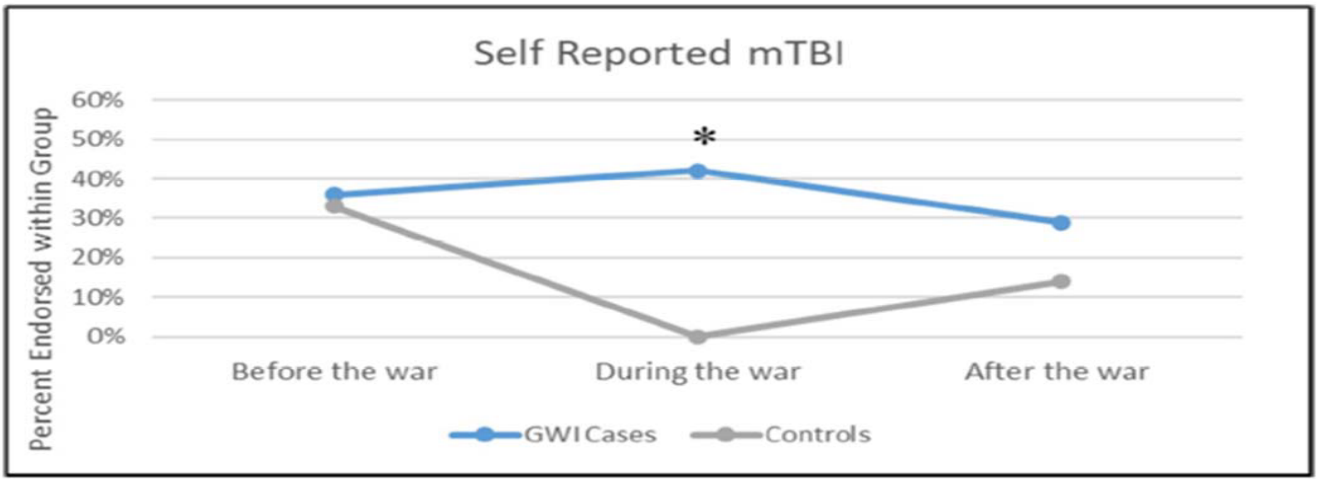


Figure 1. Percent of veterans with self-reported mTBI before, during, and after the war. * $p < 0.005$.

Task 5c. Upload neuroimaging data to BUSPH for post-processing of MR images and for data analysis.

MRI scans were obtained from the first 161 study participants have been post-processed. The Baylor site has transferred their MRI scans electronically to the Center for Biomedical Imaging at Boston University School of Medicine for post-processing. Each scan undergoes quality checking that consists of a visual inspection for the presence of noise or artifact as well as a review of scan parameters to ensure that the appropriate ones were used in the acquisition. Scans that fail the quality check are rejected by the study and remediation discussed with the appropriate site investigator. Scans that pass the quality check enter the post-processing pipeline. The first 161 scans have been through the post-processing pipeline for multivariate statistical analysis and initial correlation results are presented in the sections below. New processing pipelines are also being developed for second generation

diffusion MRI index mapping on cortical ROIs, PET data processing and pCASL processing. A doctoral student at BUMC has been assisting with the PET and resting state fMRI processing.

MRI Imaging: The scanning session include: 1) Three plane TFSE scout scan, 2) a Sense reference Scan, 3) an accelerated high resolution MPRAGE scan acquired in the sagittal plane, 4) a multi-component T2 imaging sequence acquired in the axial plane, 5) a Diffusion Tensor Scan with 32 directions acquired in the axial plane, 6) a resting state functional magnetic resonance imaging scan, and 7) a pCASL sequence obtained while the participant is at rest and 8) a High Angular Resolution Diffusion Imaging (HARDI DTI) scan.

Task 5d. Score neuropsychological tests and upload summary data to DCC for entry, cleaning and analyses.

Data from the first 186 participants has been scored and cleaned and basic means (sd) are presented in Table 2. As data is collected, quality control procedures have remained in place including double entry of data collection forms in the REDCap data collection website, built in range checks and quality control audits of all data collection by the Data Coordinating Center staff and the local BU Administrative Core neuropsychologists. Dr. Toomey also conducted biweekly conference calls to review scoring and quality control, as well as regular reviews of data entered and spot checks of any questionable data to ensure data administration and scoring integrity throughout the recruitment period. This has ensured the highest quality data available for analysis.

Validation Study #3

Study Description:

Analysis of stored blood samples from a previous large multi-site VA study of GWI (CSP#458) will be used to examine the relationships between proinflammatory cytokine markers and the behavioral symptoms associated with GWI, such as impaired memory and pain. This study will validate clinical results from the main study in an unrelated GW veteran cohort. We will access de-identified stored bloods and study data from the CSP #458. Subject data will come from GW veterans who were all originally enrolled in The National Health Survey of Gulf War Era Veterans and Their Families and who were determined to have CMI (n= 327). We will sample 100 veterans, divided equally into 50 with Khamisiyah exposure and 50 without. These two groups will be matched on CMI illness severity (mild-moderate vs. severe). The control sample will include 100 DV without CMI. The CMI group and controls will be matched to the extent possible on demographic variables.

Study Funding and Activity:

There have been no relevant abstracts or publications generated.

Update of study progress:

We finalized all paperwork for transferring funds to Hines VA and transferred those funds.

We finalized the algorithm for Gulf War Illness using the available data to most closely represent the Kansas definition. We identified the sample and were able to match sub-samples on all our planned for criteria. The final sample of 200 met criteria for a modified Kansas definition and a modified CDC definition of Gulf War Illness.

We sent the list and documentation to Boston MAVERIC. Boston MAVERIC mailed the samples to Miami this week and they arrived safely. The next step is for Dr. Klimas and her team to analyze the samples.

Task 5e. Send blood and saliva samples to Nova University for analysis of cytokine and chemokine panels and cortisol measurements.

Blood and saliva samples have been sent to NOVA Southeastern University for each of the recently completed 264 study participants. The analysis of cytokine and chemokines has been completed for the first 184 samples. Cortisol measurements that will include testing for neuroendocrine and immune alterations and for hypothalamic pituitary adrenal axis abnormalities have also been completed for the initial batched samples. Specifically, blood samples are sent to NOVA Southeastern University for analysis of proinflammatory cytokine and chemokines, and nanostring analysis of mRNA and miRNA of proteins related to TLR4 functioning and glial activation including miR-155, miR-21 and miR-146. Multiplex Quansys ELISA system will be used with an existing cytokine platform created by Dr. Klimas' research laboratory. Dr. Klimas' laboratory currently measures 16 cytokines, chemokines and immune markers in plasma. Gene expression and pathways will also be assessed using an Agilent microarray system and quantitative realtime PCR for validation of differentially expressed genes. Preliminary results of t-tests and univariate analyses of variance controlling for gender comparing cytokine, chemokine, monocyte, glutamate and lymphocytes between cases and controls indicate initial significant differences in multiple cytokines and white blood cells between the groups that are being prepared for publication. These results have stayed significant as we have added additional cases and controls (n=266) and validate our initial hypotheses that excitatory neurotransmitters and neuroinflammation are associated with GWI. Additional results of t-tests and univariate analyses of variance controlling for age and gender comparing CNS autoantibodies and excitatory neurotransmitters between cases and controls indicate that glutamate levels as well as autoantibodies for CNS proteins including glial fibrillary acidic protein (GFAP), microtubule associated tau, microtubule associated proteins (MAP-2) and myelin associated glycoprotein (MAG). CNS autoantibody results have been combined with results from other cohorts as part of a GWIC follow up study and published on the cover of Brain Sciences (Abou-Donia et al., 2020). These results validate our prior preliminary results in CNS autoantibodies in veterans with GWI and our preclinical in-vitro study results with microtubules, myelin and astrocytes in GWI animal exposure models (Abou-donia et al., 2017; Belgrad et al., 2019; Qiang et al., 2017; Rao et al., 2017).

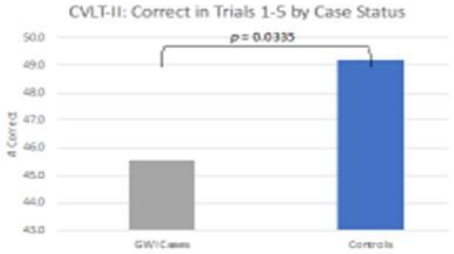
Several other investigators have reported some of the same cytokines to be different between GWI cases and controls (Parkitny et al., 2015; Zhang et al., 1999; Skowera et al., 2004; Smylie et al, 2013; Khaiboullina et al., 2015).

Preliminary MANCOVA analyses of cytokines with cognitive outcomes are also shown in the Table below. Results to date show significant differences between cases and controls on the Trail Making Test (TMT), Finger Tap Test (FTT) and the CaliforniaVerbal Learning Test II learning trials 1-5, total short-delayed recall and long delayed recall. These tests span the attention and executive system, motor and memory domains. These results are consistent with our recently published meta-analysis and review of cognitive functioning in multiple studies of GW veterans (Jeffrey et al., 2019; Janulewicz et al.,2017).

Cognitive Testing

Cognitive Test	GWI Cases Mean	Controls Mean	p-value
Trail Making Test: Trail A Time (seconds)	34.62	29.11	0.0168
Finger Tapping Test: Non Dominant Hand Mean	48.00	51.05	0.0377
California Verbal Learning Test (CVLT-II)			
# Correct in Trials 1-5	45.55	49.22	0.0335
# Correct in Short Delay Recall	9.00	10.16	0.0305
# Correct in Long Delay Recall	9.33	10.79	0.0131

*All analyses adjusted for age, gender, and years of education



Task 5f. Send additional saliva samples to University of Adelaide for genetic polymorphism analysis

As of January 2020, genomic DNA has been isolated from saliva samples of 194 Gulf War (GW) veterans with Gulf War Illness (GWI) and 33 GW veteran controls have been genotyped using the custom SNP panel (includes genetic variants of cytokines, TLR & accessory proteins and COMT). Differences between allele frequencies were compared amongst Cases and Controls and population-based data (HapMap) and the impact of genetic variability on peripheral cytokine expression, and on pain, fatigue and CPT scores was examined. Logistic regression modelling was conducted using data from Caucasian participants only to avoid erroneous associations due to differences in allelic frequencies as a result of ethnicity and not GWI. A step-up approach of model building, where each factor was added to investigate increased strength of the model indicated specific genotypes of TGF (rs1800469 SNP) occurred at significant different frequencies in GWI cases compared to controls (P = 0.011, Figure 1, where red bars indicate cases and blue bars indicate controls). More specifically, cases had a lower frequency occurrence of the wild-type genotype, C/C, and higher frequency occurrence of the heterozygote and homozygous variant genotype, C/T and T/T, compared to controls (Figure 1). In summary, this indicates that differences in TGF genetics can partially explain the development of GWI in some veterans compared to others.

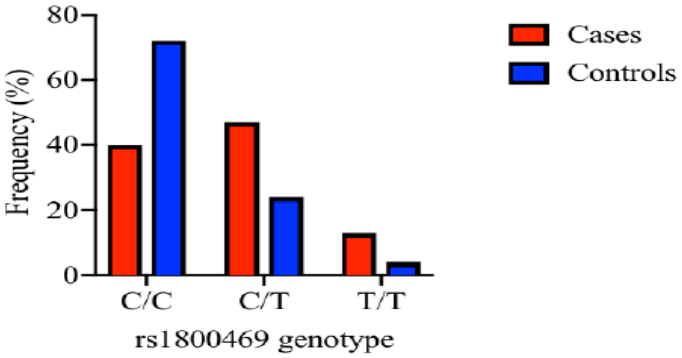


Figure 1: Genotype frequencies of TGF (rs1800469 SNP) in GWI cases (red bars) and veteran controls (blue bars). The genotype order from left to right is homozygote wild-type, heterozygote and homozygote variant.

In addition, it was observed that a SNP in the promoter of the IL10 gene, rs1800896 (-1082G/A), was related to variability in IL-10 plasma concentrations determined by an enzyme-linked immunosorbent assay (ELISA) in veterans with and without GWI. Specifically, expression was 2.3-fold lower in carriers of the homozygous wild-type genotype (G/G, median 7.07) compared to those with the homozygous variant genotype (A/A, median 16.46, P=0.031, Figure 2).

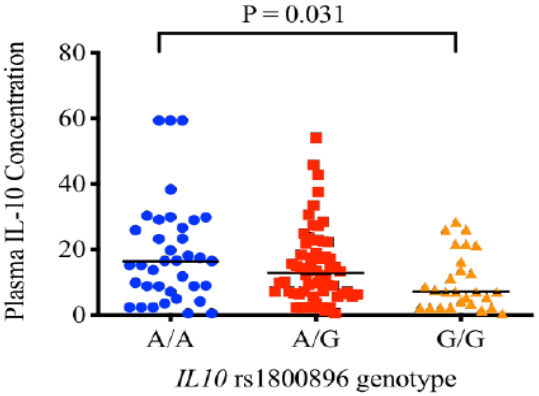
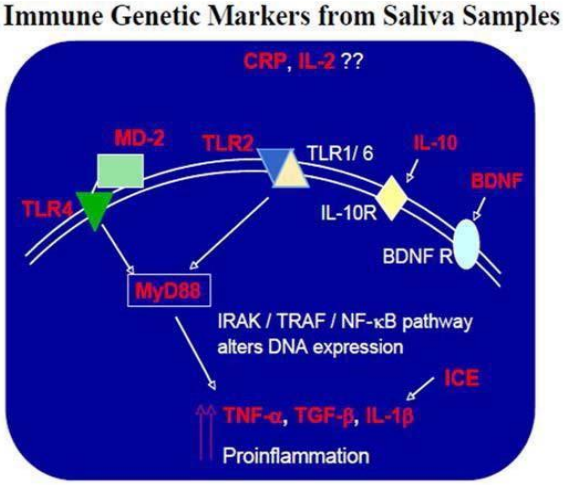


Figure 2: Plasma IL-10 concentrations (pg/mL) in GWIC participants with different genotypes of the IL-10 rs1800896 SNP; homozygous variant – A/A genotype, heterozygous – A/G genotype, and homozygous wild-type – G/G; lines are medians.



Similarly, another SNP in the promoter of the IL10 gene, rs1800871 (-891C/T), was related to variability in IL-10 plasma concentrations determined by an enzyme-linked immunosorbent assay (ELISA) in veterans with and without GWI. Specifically, expression was 1.7-fold lower in carriers of the homozygous wild-type genotype (C/C, median 9.13) compared to those carrying at least one copy of the variant allele

(C/T and T/T genotypes, median 15.84, P=0.021, Figure 3).

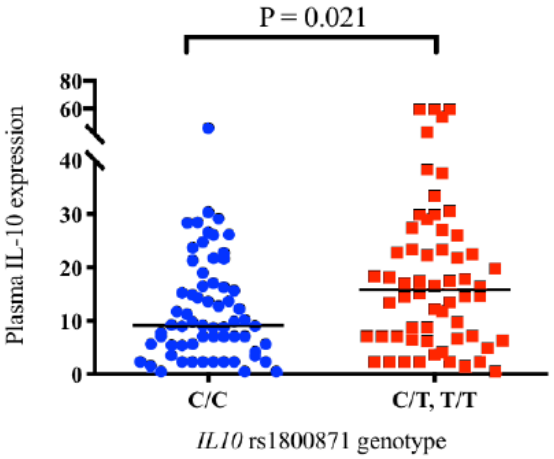


Figure 3: Plasma IL-10 concentrations (pg/mL) in GWIC participants with different genotypes of the IL-10 rs1800871 SNP; variant allele genotypes – C/T and T/T genotypes, and homozygous wild-type – C/C; lines are medians.

We have also completed preliminary mathematical modelling of the combined impact of genetics and deployment related exposures (gene-exposure associations) on the occurrence of GWI. This analysis has revealed evidence of interactive effects wherein genetic effects varied with specific exposures. For example, among veterans who used pesticide cream/spray on their skin, genetic variants in TGF-b (rs1800469 SNP) and IL6R (rs8192284 SNP) altered risk of GWI. This highlights the complex nature of interactions between potential genetic markers and deployment exposures in determining overall risk of GWI. We are yet to examine in detail all the potential interactions with deployment exposures but will do so once we have the complete cohort of samples added to the analysis using different statistical analyses including Bayesian Networking.

Task 5g. Conduct preliminary analyses of clinical data

The BUSPH Data Coordinating Center has cleaned all current data and prepared the datasets for statistical analysis from the REDCap data capture web database in direct collaboration with the study biostatistician Dr. Heeren, Dr. Sullivan and the study PIs. The overall aims of this integrated multidisciplinary consortium scientific focus are to (1) To identify validated markers of GW illness by using state of the art neuroimaging, behavioral, genetic and blood markers of neuroinflammatory activation in both clinical and preclinical models that will elucidate targeted and validated treatment strategies (2) To create a Neuroinflammation Risk Profile for GWI (3) To identify viable mechanistic treatments based on identified pathophysiological pathways of GWI that have been validated in preclinical treatment models.

Results of Pearson correlation coefficient analyses of cognitive testing outcomes compared with cytokines in GWI cases indicates significant correlations between cognitive outcomes and proinflammatory cytokines in both individual cognitive tests within different cognitive domains and by combining tests within a domain for the cognitive domains of memory, attention and executive functions, motor functions, visuospatial skills and mood functioning. For GWI cases the motor domain was correlated with TNF-alpha and the executive function domain was correlated with IL1-b ($p=<0.05$).

In terms of health symptoms, the McGill Pain inventory was correlated with IL1-a and IL1-b and Pittsburg sleep quality index was significantly correlated with TNF-RII levels. When compared with Kansas case criteria sympto m domains and cytokine outcomes, IL1-b and TNF-a were significantly correlated with fatigue, pain, neurological and skin domains and IL2 and IL4 and TNF-RI were significantly correlated with the gastrointestinal and respiratory domains. These results again suggest that treatments targeting IL1, TNF-a and other proinflammatory cytokines are viable candidates for reducing pain, fatigue and other symptoms of GWI.

Brain volumetric analysis between cases and controls showed significant differences between MRI cortex volume and precentral gyrus volume ($p<0.05$) and pars-triangularis and superior longitudinal fasciculus ($p<0.01$) when

controlling for age, gender, education and total intracranial brain volume in multivariate analyses. In addition, glutamate levels were significantly negatively correlated with many frontal gray and white matter pathways ($p<0.01$) suggesting the potential importance of excitatory neurotransmitter signaling in GWI. White matter HARDI pathways results are presented in table 2 below and show WM microstructural changes in the corpus callosum and the inferior and superior lateral fasciculus (SLF) that correspond to clinical observations clearly seen and marked on MRI scans below. SLF results correspond with those reported previously by Rayhan et al., 2013 and add strong concerns regarding additional loss of brain volumes and atrophy in superior frontal and parietal cortical areas and WM microstructural integrity in the corpus callosum and other structures. Collectively, these results suggest further quantification and validation of these potential objective diagnostic and pathogenic imaging markers of GWI.

Table 2. MRI Analyses Adjusted for Age, Gender, Years of Education and Total Intracranial Volume

Table 3. T-test Comparisons of WM Microstructural Integrity in GWI Cases vs Controls

The Boston call-back studies are now completed. The microbiome pilot study has successfully recruited 30 of 30 participants to date. This pilot study provided microbiome pilot data that resulted in two new grant submissions that were recommended for funding with Dr. Saurabh Chatterjee from University of S. Carolina and GWIC investigators. These studies will focus on gut-brain axis as a contributor to GWI and assess sodiumbutyrate as a potential new treatment for gastrointestinal and other symptoms of GWI. Drs. Chatterjee, Sullivan and Janulewicz have published a paper in Toxicology and Applied Pharmacology (TAAP) which shows reduction of neuroinflammation in GWI mice given oral sodium butyrate with no adverse outcomes in the animals at (Seth et al., 2018). Specifically, results in a GWI-mouse model showed that oral butyrate restored gut homeostasis and increased GPR109A receptor copies in the small intestine (SI). Claudin-2, a protein shown to be upregulated in conditions of leaky gut was significantly decreased following butyrate administration. Butyrate decreased TLR4 and TLR5 expressions in the liver concomitant to a decrease in TLR4 activation. Chatterjee et al., has also recently published results of virome assessments of GWI animal models suggesting another potential treatment pathway for GWI (Kimono et al., 2019). This team has also published the results from the gut microbiome pilot study with GW veterans showing altered gut micrbiome ratios in veterans with GWI and those with GWI and GI problems compared with healthy controls. These results also corresponded with blood cytokines such that veterans with GWI +/- GI problems had nearly double the amount of TNF-RI levels in their blood compared with healthy GW veteran controls. See appendix for full publication by Janulewicz et al. 2019. Three additional publications were accepted this year related to the treatment of gut-brain axis and neuroinflammation in GWI (Kimono et al., 2020; Bose et al., 2020; Bose et al., 2020).

Specific recruitment numbers, plans and updates for other call back studies are listed below.

	Total	Boston	Miami	Texas
Number of Subjects Contacted	660	382	79	199
Number of Subjects Screened	633 (95.9%)	366 (95.8%)	79 (100.0%)	188 (94.5%)
Number of Subjects Eligible	399 (63.0%)	213 (58.2%)	69 (87.3%)	117 (62.2%)
Number of Subjects with Appointments Made	280 (70.2%)	142 (66.7%)	64 (92.8%)	74 (63.2%)
Number of Subjects Assessed	264 (94.3%)	141 (99.3%)	50 (78.1%)	73 (98.6%)
Subjects Participating in Stem Cell Call-Back Study	19	19	0	0
Subjects Participating in LP Call-Back Study	10	10	0	0
Subjects Participating in PET Scan Call-Back Study	30	30	0	0
Subjects Participating in Microbiome Call-Back Study	27	27	0	0

Task 6a. Perform lumbar punctures to obtain cerebrospinal fluid markers of neuroinflammation in 50 GW Veterans

The first ten lumbar punctures have been completed but due to poor response for additional recruitment, we will now collaborate with other studies at Baylor College of Medicine (BCM) who will share their CSF samples to improve our numbers to start performing data analyses for this pilot study. IRB amendments have been submitted for this sample and data sharing and will now be sent to HRPO for approval before samples are shared with BCM investigators.

Task 6b. Perform positron emission tomography (PET) scanning with novel EAAT2 ligand in partnership with RIO pharmaceuticals in 15 GW veterans.

The PET animal pilot study has now been conducted as previously described and the final report from RIO staff is anticipated in the next quarter.

Task 6c. Perform FDG-PET scan imaging with 30 GW veterans after a computerized CPT cognitive challenge task. - Because many marker differences identified in GWI have been shown after challenge tasks, a FDG-PET imaging pilot study was conducted after a continuous performance test (CPT) of information processing and sustained attention task 30 GW veterans were assessed for differences in glucose utilization when compared with GW veteran healthy controls. Recruitment has been completed for this this pilot study and data analysis is in progress. Importantly, a new study has suggested that FDG PET signal may actually be driven by astrocytic glutamate transport as a glial contribution to neuroenergetics (Zimmer et al., 2017).

Thus, analyses will be done to compare glutamate and GFAP blood levels in participants in this pilot study to assess potential astrocyte contribution to this imaging modality. A new CDMRP grant was also submitted to assess PET imaging ligands including PBR28 (microglial), EAAT2 or deprenyl (astrocyte) and [^{18}F] FDG PET to use now available radioligands to tease out whether GWI is a predominant microglial or astrocyte induced (or both) chronic neuroinflammatory disorder. If FDG PET overlaps with and is equivalent to the other astrocyte PET ligands, it would be an easily deployable diagnostic test due to its wide availability in military and VA as well as other civilian hospitals.

Task. 7. Interim Analyses, Grant Submission, and Annual Reporting (Months 18-42)

7a. Data entry of all questionnaires, evaluations and quality control measures will be ongoing

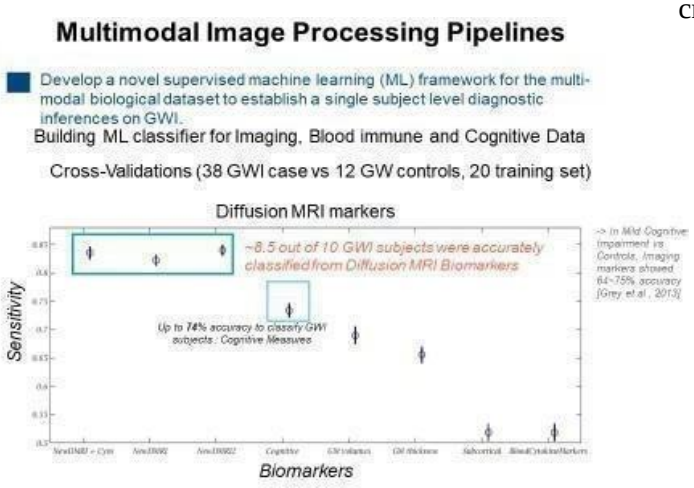
Data entry of all questionnaires and evaluations has been ongoing in as close to real time as possible. The Data Coordinating Center also tracks missing and inconsistent data. The latest data integrity report is listed below and shows very few missing or out of range data points.

GWIC: Gulf War Illness Consortium
Data Integrity Report
Data as of March 31, 2020

Site	Out of Range Data	Missing Data Fields	Invalid Logic
Boston	0.04% (34 of 81467)	0.13% (342 of 256325)	0.02% (1 of 4378)
Miami	0.03% (5 of 16214)	0.14% (72 of 50877)	0.00% (0 of 869)
Texas	0.03% (14 of 40730)	0.27% (352 of 128159)	0.09% (2 of 2189)
Total	0.04% (53 of 138411)	0.18% (766 of 435361)	0.04% (3 of 7436)

7b. Interim Statistical analyses of data obtained from cognitive evaluations, blood markers, neuroimaging and questionnaire data will be performed periodically.

Interim analyses were presented above in Task 6 and additional analyses of machine learning multi-modal approach to brain connectomics is continuing to be conducted. Results to date are very promising and show that diffusion tensor imaging of WM microstructural outcomes predict Kansas GWI cases 94% of the time. Further analyses are being conducted to improve the prediction rate further to closer to 100% GWI case classification. These results have been submitted to Brain Sciences for publication. A new CDMRP grant was submitted to include longitudinal MRI and other markers of GWI to further refine and predict GWI criteria using machine learning and other advanced data analytics.



Brain imaging studies have shown significantly lower cortical frontal pathway volumes and worse microstructural integrity of the WM pathways including the superior and inferior lateral fasciculi and the corpus callosum forceps minor that correlate with increased signaling of the excitatory neurotransmitter glutamate. GWIC structure-function relationships in GWI cases showed correlations with High Angular Resolution Diffusion Imaging (HARDI) sequences to determine WM microstructural integrity and behavioral outcomes such that as WM integrity decreased, symptom complaints of pain, fatigue and poor sleep quality increased and cognitive performance on attention/executive system functioning, motor functioning and information processing were adversely affected. Some of these results were recently published by Cheng et al., 2020 in Brain Sciences.

Dr. Little from the GWIC Houston site has observed a number of pathogical indicators on the MRI brain scans of the veterans with GWI scanned for the GWIC. Three types of tissue abnormalities were consistently observable on MRI scans of individual veterans with GWI and may have clinical utility and diagnostic potential for ill veterans. Drs. Little and Steele in Houston have collaborated with Drs. Sullivan and Killiany in Boston on a new grant proposal to quantify, validate and further expand these findings and determine their diagnostic utility.

7c. Grant submissions to relevant funding agencies for further collaborative studies based on initial results and preliminary data targeted toward treatment strategies will be ongoing.

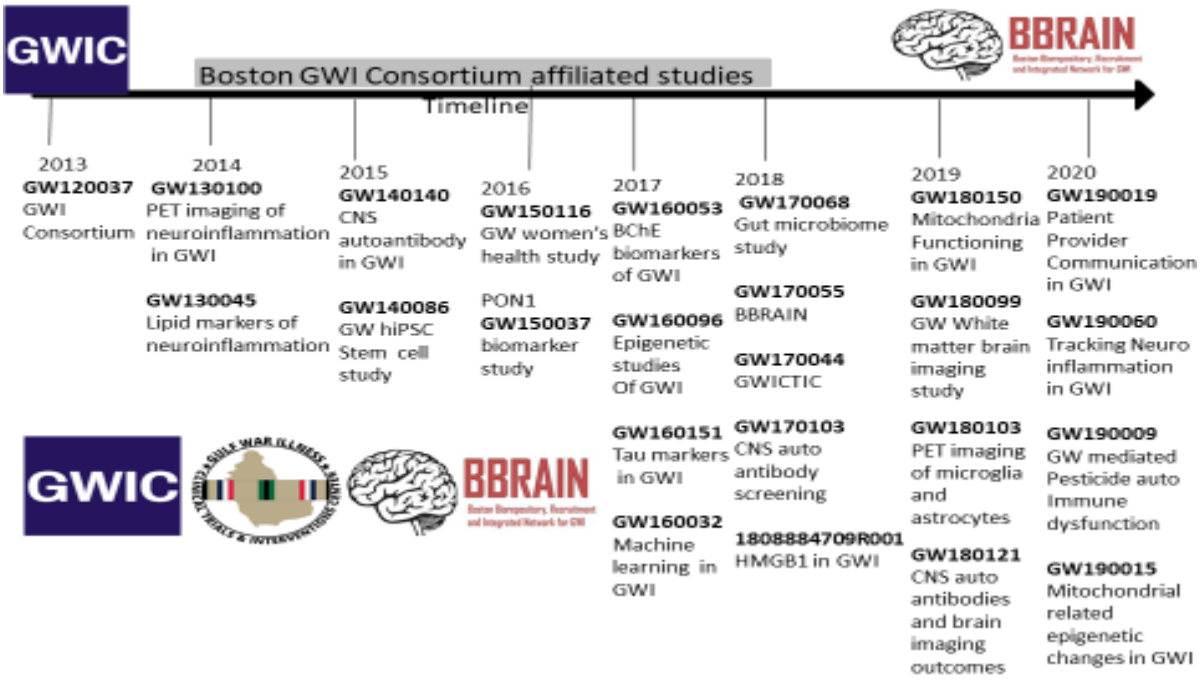
Nine letters of intent were submitted in the last quarter primarily from our GWIC senior investigators and collaborators in order to further expand scientific expertise in solving the problem of GWI biomarkers and treatment development and were invited to submit full applications in September 2020. These studies will utilize the GWIC biorepository for further analyses of biomarkers, gene-exposure outcomes and clinical treatments and we also now utilized the recently funded BBRAIN biorepository network of GWI samples. Five recently submitted grants were funded by CDMRP.

Submitted and Funded Grant Applications:

1. *Funded*- 'Patient Provider Communication Training Grant'
2. *Funded*- 'Brain-derived extracellular vesicles of neuroinflammation in blood samples'
3. *Funded* - 'Failure of Neurogenesis as a Treatable Mechanism Underlying Memory Loss in Gulf War Illness - alternate'
4. *Funded*- 'Gulf War pesticide metabolite mediated autoimmune dysfunction in Gulf War Illness'
5. *Funded* - 'Mitochondrial epigenetic changes as a biomarker for GWI and an outcome measure in response to therapy'

7d. Annual reports of progress will be written. Annual reports have been written for the past 6 years with corresponding quarterly reports in between.

Key Research Accomplishments: Thirty-two manuscripts and 30 abstracts have been published. Twenty six studies have been funded to date including two new consortia. This multi-institutional collaboration of highly qualified GWI researchers from public universities, federal agencies, and the private sector, provide an unprecedented opportunity to fully elucidate the underlying pathobiology of GWI in one integrated model that once proven, will lead to focused treatment trials that can be quickly implemented. The central hypothesis for the pathobiological mechanisms of GWI in this consortium includes chronic neuroinflammation as a result of initial glial activation and then priming of glial responses that cause stronger and longer responses that do not shut off the chemical cascade of proinflammatory cytokines and chemokines that cross-talk between the immune system and the brain. This could result in a lasting multisystem illness affecting many body systems, as seen in GWI.



Improved understanding of the role of glial activation in chronic pain states has given rise to rapidly expanding efforts to identify pharmaceuticals that specifically focus on glial functions. The growing availability of treatments of this type gives particular urgency to our efforts to determine the extent to which glial activation and central cytokine activation explain the symptoms of GWI. In order to specifically address the research gaps outlined by the IOM and the RAC reports with regard to biomarker identification and pathobiology of GWI, this research team is characterizing disease symptoms and validating and improving pathobiological markers based on collective prior clinical and preclinical studies and leveraging longitudinal cohorts and stored blood samples with the ultimate goal of identifying targeted and effective treatments for GWI. Results to date suggest that the consortium animal model of GWI is correlated with behavioral alterations seen in clinical studies including altered memory functioning and that chronic neuroinflammation and microglial and astrocyte activation are present in the DFP + CORT model. Myelin studies show an increase in myelin basic protein at early and later time points that is not associated with new myelination. Calcium signaling is also increased in oligodendrocytes in the GWI model. Microtubule stability and axonal transport are also found in animal and stem cell derived organoid GWI models. These

preclinical model results provide clear pathways for objective biomarkers and targeted treatments for GWI. Clinical results to date suggest that proinflammatory cytokines are increased in veterans with GWI and this correlates with behavioral outcomes including cognitive functioning and health symptoms of fatigue, pain and sleep problems. Brain imaging results indicate that veterans with GWI show atrophy in the frontal and parietal lobes and brain white matter. Microstructural integrity of the white matter is also impaired in veterans with GWI and correlates with proinflammatory cytokines.

Conclusion: Several promising therapeutic avenues have been developed to date in the preclinical studies and several more have been approved for ongoing pilot studies. Preliminary clinical study results suggest brain-immune-behavioral outcome correlations that bode well for the derivation of a neuroinflammatory risk profile of GWI, diagnostic marker development and targeted therapeutic strategies in the very near future. Specific results show cognitive decrements and brain imaging alterations in white matter (WM) volumes, WM integrity in Corpus Callosum and other key WM pathways and frontal and parietal gray matter volumes. These structure and functional alterations corrrelate with blood markers including cytokines and glutamate. When combining these markers into multi-modal analyses, we can predict GWI cases 95% of time. In addition, CNS autoantibody These provide key areas to focus on for objective biomarkers of GWI and focused treatment targets.

PUBLICATIONS, ABSTRACTS and PRESENTATIONS

- Jasmine Cheng, Bang-Bon Koo and Kimberly Sullivan. Diffusion Imaging features for classifying veterans with Gulf War illness with and without mTBI. International Neuropsychological Society annual meeting, New York City, February 2019.
- Bang-Bon Koo, Jasmine Cheng, Deborah M. Little, Lea Steele, Timothy Heeren and Kimberly Sullivan. mild TBI is associated with cortical gray and white matter microstructural alterations in 1991 Gulf War Veterans with Gulf War Illness. The International Brain Injury Association's World Congress on Brain Injury. Toronto, March 2019.
- Lindsay T. Michalovicz, Kimberly A. Kelly, Julie V. Miller, Diane B. Miller, Kimberly Sullivan, James P. O'Callaghan. Propranolol as a novel treatment for Gulf War Illness in a preclinical mouse model. Society of Toxicology. Baltimore, March 2019.
- Jillian Belgrad, Dipankar J. Dutta, Kimberly A. Sullivan, James P. O'Callaghan, R. Douglas Fields. Cholinergic Signaling Between Axon and Oligodendrocyte: Implications for Myelin Abnormalities in Gulf War Illness. Society for Neuroscience. Chicago, October 2019.
- Saurabh Chatterjee, Diana Kimono, Sutapa Sarkar, Ratanesh Kumar Seth, Dipro Bose, Muayad Albadrani, Ayan Mondal, Yuxi Li, Prakash Nagarkatti, Mitzi Nagarkatti, Kimberly Sullivan, Patricia Janelewicz, Ronnie Horner, Stephen Lasley, Nancy Klimas. Dysbiosis associated Enteric glial cell immunoactivation and redox imbalance modulate tight junction protein expression in gulf war illness pathology. 2019. Frontiers in physiology. Vol 10. Pages 1229.
- Bang-Bon Koo, Chia Hsin Cheng, Deborah Little, Lea Steele, Timothy Heeren, Kimberly Sullivan. Mild TBI During the War is Associated with Further Microstructural Alterations in the Cortical Gray and White Matter in 1991Gulf War Veterans with Gulf War Illness BRAIN INJURY. May 2, 2019. Page 203. TAYLOR & FRANCIS LTD.
- K. Sullivan, N. Klimas. State of the Science GWI – Moving Knowledge to Treatment (Invited keynote Speaker), Ft. Lauderdale, Florida, February 28, 2020
- K. Sullivan. The Glial Hypothesis of Gulf War Illness. School-wide Assembly.

Boston University School of Public Health, Boston, MA December 12, 2019.

- K. Sullivan. Neurotoxicity of Gulf War Deployment. Human Neuropsychology class (guest lecture), Boston University School of Medicine, Boston, MA December 5, 2019.
- K. Sullivan. Military Biorepositories. National Academy of Science (NAS) Workshop on Burn Pit Exposures (Invited Speaker), Washington, DC, October 3, 2019.
- K. Sullivan. Military Occupational Health and Toxicology. American Academy of Environmental Medicine annual meeting. (Invited keynote speaker), Louisville, KY, October 12, 2019.
- K. Sullivan. Military Occupational Health and Toxicology. Environmental Health Doctoral Seminar (guest lecture), Boston University School of Public Health, Boston, MA February 8, 2019.

Publications (see appendix for full-papers and *for those freely available)

GWIC Published Papers:

1. Seth RK, Maqsood R, Mondal A, Bose D, Kimono D, Holland LA, Janulewicz Lloyd P, Klimas N, Horner RD, Sullivan K, Lim ES, Chatterjee S. Gut DNA Virome Diversity and Its Association with Host Bacteria Regulate Inflammatory Phenotype and Neuronal Immunotoxicity in Experimental Gulf War Illness. *Viruses*. 2019 Oct 21;11(10). pii: E968. doi: 10.3390/v11100968.*

2. Joshi U, Pearson A, Evans JE, Langlois H, Saltiel N, Ojo J, Klimas N, Sullivan K, Keegan AP, Oberlin S, Darcey T, Cseresznye A, Raya B, Paris D, Hammock B, Vasylieva N, Hongsibsong S, Stern LJ, Crawford F, Mullan M, Abdullah L. A permethrin metabolite is associated with adaptive immune responses in Gulf War Illness. *Brain Behav Immun*. 2019 Oct;81:545-559. doi: 10.1016/j.bbi.2019.07.015. Epub 2019 Jul 17.

3. Janulewicz, Patricia A., Ratanesh K. Seth, Jeffrey M. Carlson, Joy Ajama, Emily Quinn, Timothy Heeren, and others, The Gut-Microbiome in Gulf War Veterans: A Preliminary Report, *International Journal of Environmental Research and Public Health*, 16.19 (2019), 3751. https://doi.org/10.3390/ijerph16193751

4. Zundel, Clara G., Maxine H. Krengel, Timothy Heeren, Megan K. Yee, Claudia M. Grasso, Patricia A. Janulewicz Lloyd, and others, Rates of Chronic Medical Conditions in 1991 Gulf War Veterans Compared to the General Population. *International Journal of Environmental Research and Public Health*, 16.6 (2019). https://doi.org/10.3390/ijerph16060949*

5. Belgrad, Jillian, Dipankar J. Dutta, Samantha Bromley-Coolidge, Kimberly A. Kelly, Lindsay T. Michalovicz, Kimberly A. Sullivan, and others, Oligodendrocyte Involvement in Gulf War Illness. *Glia*, 67.11 (2019), 2107–24. https://doi.org/10.1002/glia.23668

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7. Chatterjee, Saurabh, Kimono, Diana, Sutapa Sarkar, Muayad Albadrani, Ratanesh Seth, Dipro Bose, Ayan Mondal, and others, 'Dysbiosis-Associated Enteric Glial Cell Immune-Activation and Redox Imbalance Modulate Tight Junction Protein Expression in Gulf War Illness Pathology', *Frontiers in Physiology*, 10 (2019). https://doi.org/10.3389/fphys.2019.01229*

8. Latimer, Jean J., Abdullah Alhamed, Stefanie Sveiven, Ali Almutairy, Nancy G. Klimas, Maria Abreu, and others, 'Preliminary Evidence for a Hormetic Effect on DNA Nucleotide Excision Repair in Veterans with Gulf War Illness', *Military Medicine* (2019).

https://doi.org/10.1093/milmed/usz177

9. Michalovicz, Lindsay T., Kimberly A. Kelly, Saurabh Vashishtha, Rotem Ben-Hamo, Sol Efroni, Julie V. Miller, and others, 'Astrocyte-specific Transcriptome Analysis Using the ALDH1L1 BacTRAP Mouse Reveals Novel Biomarkers of Astrogliosis in Response to Neurotoxicity', *Journal of Neurochemistry*, 150.4 (2019), 420–40. https://doi.org/10.1111/jnc.14800

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Abstracts

DH Woo, DJ **Dutta**, W Huffman, M Robnett, PR Lee, K **Sullivan**, R **Killiany**, J **O'Callaghan**, RD **Fields:** Role of glia in the pathophysiology of Gulf War Illness. Abstract & Poster. Society for Neuroscience, San Diego, CA Oct 2016.

K. Abdullah, T. Emmerich, JE Evans, U Joshi, J Reed, G. Laco, V. Mathura, **K. Sullivan, N. Klimas, M.** Mullan, F. Crawford. Application of lipidomics for identifyingnovel blood biomarkers of Gulf War Illness. Abstract and poster. International Association of Chronic Fatigue Syndrome, Ft. Lauderdale, FL Oct 2016.

M.B. Abou-Donia**, K. Sullivan, ,** L. Conboy**,** E. Kokkotou**:** Serum Autoantibodies to Neural-Specific Proteins as Objective Biomarkers for Gulf War Illness. Abstract & Poster. Society of Toxicology, New Orleans, LA March 2016.

Kelly KA, **Locker AR,** Michalovicz LT, Miller DB, O'Callaghan JP: Exploration of the gulf war illness phenotype in a mouse model challenged with LPS at long term time points. Abstract & Poster. Society of Toxicology, New Orleans, LA March 2016.

Revitsky AR, Kelly KA, Miller DB, Lasley SM, **O'Callaghan JP**: Organophosphate-induced Neuroinflammation, With and Without Corticosterone Pretreatment, Is Not Due to Acetylcholinesterase Inhibition. Abstract & Poster. Society of Toxicology, New Orleans, LA March 2016

Michalovicz LT, **Locker AR, Kelly KA**, Miller DB, **O'Callaghan JP:** Corticosterone priming of the neuroinflammatory response to acetylcholinesterase inhibitors results in overexpression of TLR2 and downstream targets, but not activation of the NLRP3 inflammasome. Abstract & Poster. Society of Toxicology, New Orleans, LA March 2016

Yee, M., Seichepine, D., **Janulewicz Lloyd P., Sullivan, K. & Krengel,** M. History of Pre-War Brain Injuries Influences Total Current Health Symptoms in a Cohort of 1990-1991 Gulf War Veterans. Abstract. 43rd Annual Meeting Abstracts, Journal of the International Neuropsychological Society, Supplement 1, March 2015: 8.

Seichepine, D., Yee M., **Janulewicz Lloyd P., Sullivan, K. & Krengel, M.** Frequency of Traumatic Brain Injuries in a Cohort of 1990-1991 Gulf War Veterans. Abstract. 43rd Annual Meeting Abstracts, Journal of the International Neuropsychological Society, Supplement 1, March 2015: 14.

Maule, A., **Janulewicz, P., Krengel, M.,** White, RF, Judd, S., Cirillo, J., **Sullivan, K**. A meta-analysis of Self-reported Neurological and Neuropsychological Symptoms in Gulf War Veterans. Journal of the International Neuropsychological Society, Supplement 1, March 2015: 9.

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Michalovicz LT, **Locker AR, Kelly KA,** Miller DB, **O'Callaghan JP**: Chronic corticosterone primes the brain response to select neuroinflammatory agents by overexpression of toll-like receptor 2 and S100A8: A potential role of microglia. Abstract & Poster. Society for Neuroscience, Chicago, IL October 2015

Locker AR, Kelly KA, Michalovicz LT, Miller DB, **O'Callaghan JP:** Corticosterone primes the neuroinflammatory responses to Gulf War Illness associated exposures: Effects of irreversible vs reversible acetylcholinesterase inhibitors. Abstract & Poster. Society for Neuroscience, Chicago, IL October 2015

Kelly KA, Locker AR, Michalovicz LT, Miller DB, **O'Callaghan JP:** Phenotype comparisons of ALDH1L1 BAC-TRAP mice under control and neurotoxic (MPTP) conditions. Abstract & Poster. Society for Neuroscience, Chicago, IL October 2015

Revitsky AR, Kelly KA, Miller DB, Lasley SM, **O'Callaghan JP**: Pyridostigmine bromide suppresses neuroinflammation induced by DFP. Abstract & Poster. Society of Toxicology, San Diego, CA March 2015

Kelly KA, Revitsky AR, Miller DB, Lasley SM, **O'Callaghan JP**: Chronic glucocorticoid and nerve agent DFP exposures produce a neuroinflammatory model of Gulf War Illness without neurodegeneration. Abstract & Poster. Society of Toxicology, San Diego, CA March 2015

Presentations

K. Sullivan. The Boston Gulf War Illness Consortium. 30th Anniversary of Operation Desert Storm, GWI State of the Science meeting (virtual), August 18, 2020.

K. Sullivan, N. Klimas. State of the Science GWI – Moving Knowledge to Treatment **(*Invited keynote Speaker*),** Ft. Lauderdale, Florida, February 28, 2020.

K. Sullivan. The Glial Hypothesis of Gulf War Illness. School-wide Assembly. Boston University School of Public Health, Boston, MA December 12, 2019.

K. Sullivan. Neurotoxicity of Gulf War Deployment. Human Neuropsychology class (guest lecture), Boston University School of Medicine, Boston, MA December 5, 2019.

K. **Sullivan.** Military Biorepositories. National Academy of Science (NAS) Workshop on Gulf War Respiratory Health Committee (Invited Speaker), Washington, DC, October 3, 2019.

K. **Sullivan.** Military Occupational Health and Toxicology. American Academy of Environmental Medicine annual meeting. (Invited keynote speaker), Louisville, KY, October 12, 2019.

N. Klimas. Can We "Reboot" Human Homeostasis to Cure Chronic Illness? What We Are Learning from Gulf War Illness and ME. American Academy of Environmental Medicine annual meeting. (Invited keynote speaker), Louisville, KY, October 12, 2019.

K. **Sullivan**. Understanding Gulf War Illness: Brain-Immune Biomarkers, Cognitive Functioning and Treatment Development Strategies 25 Years after the War. Symposium. International Neuropsychological Society (INS) in London, England July 6th- 8th 2017.

K. **Sullivan**. Brain Immune Interactions in Gulf War Illness: Cytokines and Cognition in US Military Veterans. Symposium. International Neuropsychological Society (INS) in London, July 6th- 8th

K. **Sullivan** Cytokines, Cognition and Gulf War Illness in a US Military Veteran Cohort. Symposium. International Neuropsychological Society (INS) in London, July 6th- 8th

Golier, J. A Controlled Trial of a Glucocorticoid Receptor Antagonist in Gulf War Veterans with Chronic Multisymptom Illness. Symposium. International Neuropsychological Society (INS) in London, July 6th- 8th

Abou-Donia M. Screening for novel objective central nervous system biomarkers in veterans with Gulf War

Illness. Symposium. International Neuropsychological Society (INS) in London, July 6th- 8th

Krengel, M. Exploring the association between cognitive symptoms and exposures in a cohort of 1990-1991 US Gulf War Veterans. Symposium. International Neuropsychological Society (INS) in London, July 6th- 8th

Meggs, W. Double-blinded Placebo-Controlled Cross-over Pilot Trial of Naltrexone to Treat Gulf War Illness. Symposium. International Neuropsychological Society (INS) in London, July 6th- 8th

Sullivan, K. Neurotoxicity of Gulf War Deployment: The Neuropsychological and Neuroimaging Correlates. Boston University School of Public Health, Introduction to Toxicology (EH768, guest lecture), Boston, MA, March, 16, 2016.

Sullivan, K., Klimas, N. Committee and Panel Discussion: 'how to discussion' for GWI Biomarker Research, Research Advisory Committee on Gulf War Veterans' Illnesses; Spring Meeting, Washington, DC, September, 2014.

O'Callaghan, J., **Sullivan, K.** Committee and Panel Discussion: 'how to discussion' for GWI animal research, Research Advisory Committee on Gulf War Veterans' Illnesses; Spring Meeting, Washington, DC, April, 2014.

Seichepine, D., Yee M., **Janulewicz Lloyd P**., **Sullivan, K.** & **Krengel, M**. Chronicity of Health Symptoms in the Ft. Devens Cohort. International Neuropsychological Society, 42nd Annual Meeting, Seattle, WA, February 2014.

Steele, L. Committee and Panel Discussion: 'how to discussion' for GWI Case Criteria Research Advisory Committee on Gulf War Veterans' Illnesses; Winter Meeting, Washington, DC, January, 2014.

Seichepine, D., Yee, M., **Janulewicz Lloyd P., Sullivan, K.**, Proctor, S., & **Krengel, M.** Traumatic Brain Injury and Health Status of Veterans from the 1990-1991 Gulf War. Boston University Second Annual Joining Forces TBI/PTSD Event, Boston, MA, December, 11, 2013.

Sullivan, K. RAC-GWVI Treatment Development Discussion. Research Advisory Committee on Gulf War Veterans' Illnesses; Summer Meeting, Washington, DC, June, 2013.

Sullivan, K. Neurotoxicity of Gulf War Deployment: The Neuropsychological and Neuroimaging Correlates. Boston University School of Public Health, Introduction to Toxicology (EH768, guest lecture), Boston, MA, March, 26, 2013.

INVENTIONS, PATENTS AND LICENSES –

REPORTABLE OUTCOMES

Current/Newly Funded Studies

- Identifying Gaps in Patient Provider Communication and Improving Care for Veterans with Gulf War Illness (PI: Krengel) (GW190119)
- Tracking Neuroinflammation in GWI from Brain Derived Extracellular Vesicles (PI: Shetty) (GW190060)
- Gulf War Pesticide Metabolite Mediated Autoimmune Dysfunction (PI: Abdullah) (GW190009)
- Mitochondrial Epigenetic Changes as a Biomarker for GWI and an Outcome Measure in Response to Therapy (PI: Ait-Ghezalia)(GW190015)
- Clarifying the Role Played by Microglia and Astrocyte Activation in Veterans with Gulf War Illness Using Positron Emission Tomography (PET) (PI: Killiany)(GW180103)
- Identifying Objective Diagnostic Markers of Gulf War Illness: Salivary and Plasma Autoantibodies Against Neural Proteins Validated With Brain Imaging (PI: Abou Donia) (GW180121)
- Defining and Characterizing GWI Pathobiology using Longitudinal Brain Imaging Biomarkers of White Matter Integrity and Hemodynamic Response (PI: Sullivan) (GW180099)
- Boston Biorepository and Integrative Network for Gulf War Illness (BBRAIN) (PI: Sullivan; GW170055)
- The Gulf War Illness Clinical Trials and Interventions Consortium (GWICTIC) (PI: Klimas; GW170044)
- Microtubule-Based Therapy for Neurodegeneration in Gulf War Illness: Studies with hiPSC-Derived Neurons from Gulf War Veterans (PI: Baas; GW170033)
- Novel Combinatorial screening for Neurotrophins, Neuropoietic cytokines, Matrix Metalloproteinases and Complement components in relevance to Neuronal Autoantibodies in the serum and CSF of Veteran with Gulf War illness (PI: Mulugu; GW170103)
- Computer Aided Decoding of Brain-Immune Interactions in Gulf War Illness (GWI): A Joint Embedding on Brain Connectomic and Immunogenetic Markers (PI: Koo; GW160032)
- BChE + PON1 biomarker epidemiological New Investigator proposal (PI: Janulewicz; GW160053)
- Epigenetic DNA methylation study with Naval Research Lab investigators (PI: Malanoski; GW160096)
- B-cell depletion Rituximab treatment trial proposal with NSU investigators (PI: Klimas; GW160123)
- Tau pathology as a contributor to GWI (PI: Qiang; GW160151)
- PET PBR28 study funded with MGH investigators (Loggia: PI; GW130100)
- Human induced pluripotent stem cells (IPSC) stem cell grant funded with Drexel and BU investigators (Baas PI; Sullivan site PI; GW140086)
- D-cycloserine pilot treatment study funded with Boston University investigators (Toomey PI; Sullivan co-I) (GW140069)
- CNS autoantibody grant with Duke investigators (Sullivan Initiating PI; Abou Donia Partnering PI; GW140140)
- CoQ10 Phase III trial, 4 site study submitted to VA with Miami VA, GWIC and other investigators Ft Devens cohort cognitive, blood and neuroimaging assessment of brain antioxidant glutathione levels (Krengel, Initiating PI; Sullivan Partnering PI; GW150050)
- PON1 study with GWIC investigators and San Francisco VA investigators GW150037)
- Gulf War Women's Health Cohort with Augusta University investigators (GW150116)
- Lipidomics and proteomics study with Roskamp Institute investigators (GW150056)
- +naltrexone pain treatment New Investigator proposal with U-Colorado investigators (GW150187)

Newly Funded Studies

Title: Defining and Characterizing GWI Pathobiology using Longitudinal Brain Imaging Biomarkers of White Matter Integrity and Hemodynamic Response (PI: Sullivan)
Supporting agency: Department of Defense (CDMRP/GWIRP GW180099)
Performance period: 09/15/19 – 08/31/22
Level of funding:

Brief description of project's goals/Specific aims: The study objectives are to confirm, validate and further define white matter (WM) microstructural integrity decrements in multiple imaging modalities (DKI and HARDI). This proposal also aims to assess the overlap of WM decrements with cerebral blood flow (CBF) alterations (pCASL) in GWI cases vs controls. Specific Aims include: 1) To compare, validate and further refine the pathobiology of WM microstructural pathways and crossing fibers in GWI 2) To compare longitudinal patterns of brain volumetric, microstructural and CBF differences in 50 GWI cases and 50 healthy control veterans. Aim 3: To perform machine learning advanced analytic data reduction analyses on GWIC and BBRAIN datasets to predict GWI case status and symptom severity over time.

Title: Clarifying the Role Played by Microglia and Astrocyte Activation in Veterans with Gulf War Illness Using Positron Emission Tomography (PET) (PI: Killiany)
Supporting agency: Department of Defense (CDMRP/GWIRP GW180103)
Performance period: 10/01/19 – 09/30/22
Level of funding:

Brief description of project's goals/Specific aims: The primary objective of project is to assess the role played by Microglia and Astrocyte Activation in Gulf War Illness. The two group of subjects (10 GWI cases, 10 GW controls) will undergo a Fluorodeoxyglucose [18F] (FDG) PET scan at Boston Medical center and a second PET scan for astrocytes ([11C]-l-Deprenyl ligand) at Massachusetts General Hospital on their 3T MRI/PET scanner. GWI cases will be asked to undergo a third PET scan, this one using the ligand for microglia [11C]PBR28. Putting these data together will provide us with a better understanding of the role played by activated astrocytes in GWI and how readily this can be assessed using standard equipment and PET ligands that are readily available. Further, information from the [11C]PBR28 PET ligand binding will determine how much of the binding in the [11C]-l-Deprenyl may be coming from microglia activation.

Title: Identifying Objective Diagnostic Markers of Gulf War Illness: Salivary and Plasma Autoantibodies Against Neural Proteins Validated With Brain Imaging (PI: Abou Donia)
Supporting agency: Department of Defense (CDMRP/GWIRP GW180121)
Performance period: 9/30/19 – 9/29/22
Level of funding:

Brief description of project's goals/Specific aims: The project's specific aims are to (1) compare the levels of IgG-class autoantibodies for central nervous system (CNS) markers in the saliva, serum, and plasma of veterans with Gulf War illness (GWI) (100 cases) against healthy Gulf War (GW) veteran controls (50 controls); (2) compare and correlate brain volumetric and microstructural alterations on brain imaging with results of CNS autoantibodies in serum, plasma, and saliva from veterans with GWI and controls; (3) compare the levels of IgG-class autoantibodies for CNS markers in the blood, serum, and plasma; (4) determine the levels of IgG-class autoantibodies for CNS markers in the blood serum, plasma, and saliva of GWI cases with chronic fatigue syndrome (CFS) (50 cases) compared to GWI cases without CFS and healthy GW veteran controls (50 controls); and (5) compare and correlate brain volumetric and microstructural alterations on brain imaging with results of CNS autoantibodies in serum, plasma, and saliva from veterans with GWI and CFS compared with GWI only and healthy GW veteran controls.

Title: Boston Biorepository, Recruitment and Integrative Network (BBRAIN) for GWI (PI: Sullivan)
Supporting agency: Department of Defense (CDMRP/GWIRP GW170055)
Performance period: 9/01/18 – 8/31/21
Level of funding:

Brief description of project's goals/Specific aims: The primary objective of BBRAIN is to establish a retrospective and prospective biorepository network for GWI research by data mining from existing BBRAIN collaborator specimens and for recruiting 500 additional repository samples. The four prospective recruitment resource sites will include Boston, Miami, Bronx and San Francisco. The BBRAIN structure will provide centralized cataloguing and coordination of retrospective biorepository samples from 10 collaborating institutions who will share existing blood plasma, sera, PBMCs, cerebrospinal fluid, human-induced pluripotent stem cells (hiPSCs), DNA and saliva samples. Corresponding cognitive outcomes, brain imaging, demographics and health symptom surveys will be included in BBRAIN network datasets to allow for the comparison of biomarkers with behavioral outcomes.

Title: The Gulf War Illness Clinical Trials and Interventions Consortium (GWICTIC) (PI: Klimas)
Supporting agency**:** Department of Defense (CDMRP/GWIRP GW170044)
Performance period**:** 9/01/18 – 8/31/22
Level of funding:
Brief description of project's goals/Specific aims: This consortium aims to unify the expertise that has been developed through past CDMRP funding of GWICs based at NSU and BU, and build on their integrated research findings to implement early phase clinical trials of interventions targeting neuro-inflammation, previously identified biologic markers of disease activity and mechanisms of homeostatic reset. The infrastructure established in this proposal will thus facilitate a rapid and effective approach to evaluating potential interventions through early-phase studies and identifying promising candidates for phase III study. Specifically, study 1 (phase 1) and study 2 (phase II) will evaluate a combination approach using entanercept, an anti-TNF agent, and mifepristone, a synthetic steroid with anti-progesterone and anti-glucocorticosteroid effects. Study 3 and 4 in the phase 1 will compare CoQ10 to glutathione ability to correct CNS oxidative stress, the phase 2 takes the antioxidant with the best CNS effect and combines it with intranasal insulin. Lastly, study 5 will evaluate a nutraceutical, Bacopa, that has been shown to have multiple impacts on inflammatory cytokines and mitochondrial function.

Title: Microtubule-Based Therapy for Neurodegeneration in Gulf War Illness: Studies with hiPSC-Derived Neurons from Gulf War Veterans (PI: Baas)
Supporting agency: Department of Defense (CDMRP/GWIRP GW170023)
Performance period: 9/01/18 – 8/31/21
Level of funding:
Brief description of project's goals/Specific aims: The primary objective of this study is to establish a High throughput treatment development pipeline using hiPSC cell lines derived from Gulf War veterans and using animal models to test HDAC and kinesin 5 inhibitors and the antioxidant Co-Q10.

Title: Novel Combinatorial screening for Neurotrophins, Neuropoietic cytokines, Matrix Metalloproteinases and Complement components in relevance to Neuronal Autoantibodies in the serum and CSF of Veteran with Gulf War illness (PI: Mulugu)
Supporting agency: Department of Defense (CDMRP/GWIRP GW170103)
Performance period: 9/01/18 – 8/31/21
Level of funding:
Brief description of project's goals/Specific aims: The primary objective of this study is to establish Biomarkers of GWI that build on prior work to generate autoantibodies to CNS proteins in the blood of GW veterans by assessing additional CNS biomarkers in blood samples from GW veterans including neurotrophins, neuropoitetic cytokines and matrix metalloproteinases to provide new biomarker avenues for GWI diagnostics and therapeutics.

Title: Examination of Neuroimaging, Cognitive Functioning, and Plasma Markers in a Longitudinal Cohort of Gulf War Deployed Veterans: The Fort Devens Cohort Supporting agency: DoD/CDMRP (GW150050P1)
Performance Period: 09/30/16 – 9/29/19
Level of funding**:**

Brief description of project's goals/ Specific aims: The goal of this study is to develop brain imaging and peripheral blood plasma biomarkers of oxidative stress that correlate with cognitive and health symptom outcomes in the longitudinally followed Ft. Devens cohort of Gulf War veterans. The specific aims are (1) to conduct follow- up longitudinal cognitive evaluations on 150 GW veterans and (2) to determine, in 100 GW veterans, cross- sectional blood and neuroimaging biomarkers of glutathione metabolite (GSH) oxidative stress markers that will be correlated with cognitive and imaging outcomes.

Title: Examination of plasma PON1 paraoxonase activity and genotype in Gulf War Veterans (PI: Chao)
Supporting agency: DoD/CDMRP (GW150037)
Performance period: 09/30/16 – 9/29/19
Level of funding:
Brief description of project's goals/ Specific aims: The goal of this study is to evaluate the extent to which paraoxonase (PON1), a human enzyme that can hydrolyze the active metabolites of several organophosphorus (OP) compounds and Gulf War (GW)-related exposure interactions contribute to the risk for developing Gulf War Illness (GWI) in a large (> 800) sample of GW veterans by leveraging existing PON1 paraoxon activity and PON1192 genotype data and GW-related exposure data in 4 independent cohorts of GW veterans.

Title: Gulf War Women's Health Cohort (PI: Coughlin)
Supporting agency: DOD/CDMRP (GW150116)
Performance Period: 09/30/16 – 9/29/19
Level of funding:
Brief description of project's goals/ Specific aims:The goal of this study is to develop a large (>900) cohort of Women Gulf War veterans from prior studies, resurvey them and determine differences bet An in-vivo investigation of Brain Inflammation ween men and women Gulf War veterans' health outcomes.

Title: Examination of Neuroimaging, Cognitive Functioning, and Plasma Markers in a Longitudinal Cohort of
Gulf War Deployed Veterans: The Fort Devens Cohort (Krengel PI; Sullivan PI)
Supporting agency: DoD/CDMRP (GW150050P1) Performance Period: 09/30/16 – 9/29/19
Level of funding:
Brief description of project's goals/ Specific aims: The goal of this study is to develop brain imaging and peripheral blood plasma biomarkers of oxidative stress that correlate with cognitive and health symptom outcomes in the longitudinally followed Ft. Devens cohort of Gulf War veterans. The specific aims are (1) to conduct follow-up longitudinal cognitive evaluations on 150 GW veterans and (2) to determine, in 100 GW veterans, cross-sectional blood and neuroimaging biomarkers of glutathione metabolite (GSH) oxidative stress markers that will be correlated with cognitive and imaging outcomes.

Title: A Randomized, Double-blind Placebo-controlled Phase III Trial of Coenzyme Q10 in Gulf War Illness. (PI: Klimas)
Supporting Agency: Department of Veterans Affairs
Performance Period: 04/1/17 – 1/31/20
Level of funding:
Project Description: The goal of this study is to perform a phase III treatment trial of Co-enzyme Q10 in Gulf War veterans at four sites around the country.

Title: Computer-Aided Decoding of Brain-Immune Interactions in Gulf War Illness (GWI): A Joint Embedding on Brain Connectomic and Immunogenomic Markers (PI: Koo)
Supporting Agency: Department of Defense/CDMRP GW160032
Performance Period: 09/1/17 – 8/31/20
Level of funding:
Brief description of project goals/specific aims: The purpose of the proposed study is to apply a novel classification framework based on a combination of brain connectomics and immunoproteomics to the GWI Consortium (GWIC) database in order to develop a computerized diagnostic system for GWI. The project's 3

specific aims are (1) to build a unimodal classifier per each biological measure of GWI, including neuroimaging as well as central (CSF) and peripheral (blood) markers; (2) to decode brain-immune interactions based on joint embedding of the multidimensional classification features; and (3) to build multimodality classifiers of subtle symptom clusters in addition to the overall GWI definition (eg, the Kansas definition).

Title: Investigating Gene-Environment Interactions in Multiple Cohorts of 1990-1991 Gulf War Veterans (PI: Janulewicz)
Supporting Agency: Department of Defense/CDMRP GW160053
Performance Period: 09/1/17 – 8/31/20
Level of funding:
Brief description of project goals/specific aims: The project's specific aims are to (1) determine associations among GWI, as defined by Kansas and CDC case definitions, butyrylcholinesterase (BChE) genotype, and cholinergic exposures encountered during deployment in 4 independent cohorts of 1990-1991 Gulf War veterans (GWVs) comprising a total sample of 834 veterans; and (2) determine the association between GWI, as defined by Kansas and CDC case definitions, and exposure to cholinergic compounds in subgroups of veterans defined by specific combinations of BChE and PON1192 genotypes.

Title: Identification of Epigenetic Signatures as Biomarkers of Gulf War Illness (PI: Malanoski)
Supporting Agency: Department of Defense/CDMRP GW160096
Performance Period: 10/1/17 – 9/30/20
Level of funding:
Brief description of project goals/specific aims: Specific Aim 1: Identify Lymphocyte (leukocyte) DNA Methylation patterns specific to GWI Specific Aim 2: Discover the changes in microRNA profiles associated with GWI Specific Aim 3: Apply high performance computing bioinformatics to characterize GWI pathogenesis.

Other Achievements.

The consortium website (http://sites.bu.edu/gwic) and social media pages are continually updated to disseminate news about new papers and studies related to Gulf War Illness. Multiple news media stories have highlighted GWIC work this past year and are included on the GWIC website.

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APPENDICES

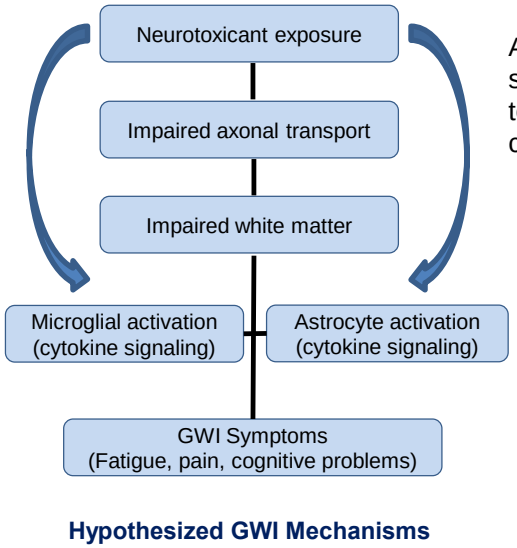
Appendix A – Quad Chart

Appendix B. Recent GWIC publications.

Brain Immune Interactions as the Basis of Gulf War Illness: Gulf War Illness Consortium (GWIC)

Award Number: GW120037 / W81XWH-13-2-0072

PI: Dr. Kimberly Sullivan **Org:** Boston University Medical Campus **Award Amount:** $4,888,851

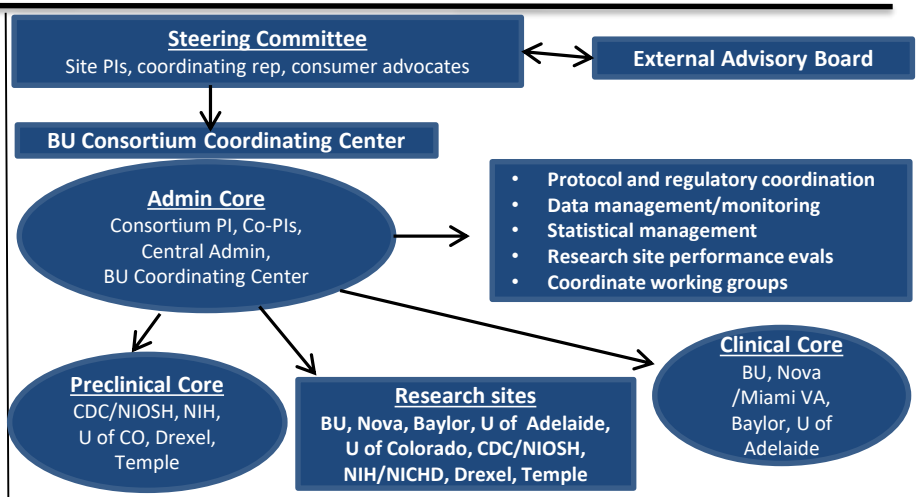


Hypothesized GWI Mechanisms

Approach

A series of clinical and preclinical studies to test whether GWI is related to chronic brain-immune activation and chronic inflammation.

- Clinical case-control studies will be conducted in parallel at 3 sites — Boston, Miami, and Central Texas and will include a total of 300 Gulf War veterans.
- Markers in blood, cerebrospinal fluid, brain imaging (advanced MRI, PET scans) and memory testing will be examined.
- Parallel preclinical studies will evaluate persistent effects of GW neurotoxicants in *in vitro* and rodent models of GWI.



Accomplishments: **1-**IACUC and ACURO approvals are in place for all sites **2-**IRB and HRPO approval obtained from BU, Baylor, Miami VA and NOVA. **3-** Data and tracking systems, websites finalized **4-**Laboratory methods established for immunologic assays. **5-** Preclinical studies ongoing at all sites, have initial results and pilot treatments started. **6-** Subject recruitment ongoing and first 267 subjects completed. **7-** Twenty six additional grant applications were funded for further collaborative research efforts. **8** -Conference symposia, 32 abstracts and 32 manuscripts published, multiple news stories published.

Timeline

Sept 2013 Start

Year

Year	1			2			3			4			Total
Months	1-4	5-8	9-12	13-16	17-20	21-24	25-28	29-32	33-36	37-40	41-44	45-48	
Task1													
Task 2													
Task 3													
Task 4													
Task 5			50	30	30	25	33	33	34	55	0		n=300 human subjects
Task 6						15	20	20	20	15	0		n=90 human subjects
Task 7													
Task 8													

Goals/Milestones

FY13 Goal – Obtain necessary authorization prior to human/animal studies and preparation for consortium clinical/preclinical studies

- ☑ Protocol preparation and initiation of approvals for animal/human use **(Task 1)**
- ☑ Creation of databases/manuals and data use agreements **(Task 2)**
- ☑ Prepare rodent dosing models and in vitro cell models **(Task 3)**

FY14 Goal –

- ☑ Perform preclinical cell/animal studies **(Task 4)**
- ☑Screening, recruitment, assessment of GW veterans at 3 sites **(Task 5)**

FY15 Goal – Recruitment and assessment for Boston CSF/PET studies ☑**(Task 6-7)**

- ☑ **FY16/17 Goal –** Statistical and validation analysis **(Task 7-8)**
- ☑ **FY17/18 Goal -** Publications and grants submissions **(Task 7-8)**

Next External Advisory Board meeting scheduled for May 2018

Brain, Behavior, and Immunity 89 (2020) 281–290

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Alterations in high-order diffusion imaging in veterans with Gulf War Illness is associated with chemical weapons exposure and mild traumatic brain injury

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ARTICLE INFO

Keywords:
Gulf War Illness
Veterans
Cytokines
Neuroinflammation
Diffusion imaging
MRI

ABSTRACT

The complex etiology behind Gulf War Illness (GWI) has been attributed to the combined exposure to neurotoxicant chemicals, brain injuries, and some combat experiences. Chronic GWI symptoms have been shown to be associated with intensified neuroinflammatory responses in animal and human studies. To investigate the neuroinflammatory responses and potential causes in Gulf War (GW) veterans, we focused on the effects of chemical/biological weapons (CBW) exposure and mild traumatic brain injury (mTBI) during the war. We applied a novel MRI diffusion processing method, Neurite density imaging (NDI), on high-order diffusion imaging to estimate microstructural alterations of brain imaging in Gulf War veterans with and without GWI, and collected plasma proinflammatory cytokine samples as well as self-reported health symptom scores. Our study identified microstructural changes specific to GWI in the frontal and limbic regions due to CBW and mTBI, and further showed distinctive microstructural patterns such that widespread changes were associated with CBW and more focal changes on diffusion imaging were observed in GW veterans with an mTBI during the war. In addition, microstructural alterations on brain imaging correlated with upregulated blood proinflammatory cytokine markers TNFRI and TNFRII and with worse outcomes on self-reported symptom measures for fatigue and sleep functioning.

Taken together, these results suggest TNF signaling mediated inflammation affects frontal and limbic regions of the brain, which may contribute to the fatigue and sleep symptoms of the disease and suggest a strong neuroinflammatory component to GWI. These results also suggest exposures to chemical weapons and mTBI during the war are associated with different patterns of peripheral and central inflammation and highlight the brain regions vulnerable to further subtle microscale morphological changes and chronic signaling to nearby glia.

1. Introduction

About a third of the nearly 700,000 U.S. troops who served in the Gulf War (GW) suffer from a complex, often debilitating symptomatic illness known as Gulf War Illness (GWI) (White et al., 2016). Symptoms of GWI typically include fatigue, chronic pain, memory and attention

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https://doi.org/10.1016/j.bbi.2020.07.006
Received 6 March 2020; Received in revised form 11 June 2020; Accepted 8 July 2020
Available online 31 July 2020

problems, headaches, gastrointestinal and respiratory symptoms which encompass the six symptom domains of the National Academy of Sciences recommended Kansas GWI criteria (Steele, 2000; National Academics of Sciences, Engineering, and Medicine, 2016). GWI has been associated with altered central nervous system (CNS) functioning (White et al., 2016). Chronic GWI symptoms are thought to develop as a result of a heightened innate immune response in the CNS to multiple exposures during the war including stress, neurotoxicant chemicals (organophosphate pesticides and nerve agents) and to other CNS insults, such as mild traumatic brain injury (mTBI) (Gade and Wenger, 2011; O'Callaghan et al., 2015; Rathbone et al., 2015; Yee et al., 2016; Yee et al., 2017; Janulewicz et al., 2018). mTBI as defined by the American Academy of Neurology has proven to be the most sensitive measure of mTBI in prior GWI research (Vynorius et al., 2016; Yee et al., 2016; Yee et al., 2017; Janulewicz et al., 2018). As such, the persisting symptoms of GWI have been hypothesized to coincide with a heightened, chronic neuroinflammatory reaction observed in animal models while increased blood levels of proinflammatory cytokines in veterans with GWI has also been reported (Whistler et al., 2009; O'Donovan et al., 2015, O'Callaghan et al., 2015; Khaiboullina et al., 2014; Parkitny et al., 2015; Locker et al., 2017; Koo et al., 2018; Miller et al., 2018; Janulewicz et al., 2019). However, we are not aware of any publications to date examining microstructural integrity and neuroinflammatory responses by utilizing brain imaging techniques to focus on mTBI and organophosphate (OP) exposure in GWI veterans.

Exposure to neurotoxicants including OP pesticides and sarin nerve agents has been a unique risk factor associated with GWI (Golomb, 2008; White et al., 2016; Sullivan et al., 2017). In animal models, exposure to OP nerve agents and pesticides, such as sarin and its surrogate diisopropyl fluorophosphate (DFP) and chlorpyrifos, was shown to produce neuroinflammation as indicated by increased proinflammatory cytokine signaling in the brain (Spradling et al., 2011; O'Callaghan et al., 2015; Locker et al., 2017). Neuroinflammatory cytokines were further associated with microstructural changes in the brain in the OP-exposed animal model of GWI indicating potential damage associated signaling and activation of proinflammatory cytokine release from nearby glia (Banks and Lein 2012; Koo et al., 2018).

These microstructural changes need not reflect neuronal damage or apoptosis but could rather reflect more subtle microscale morphological changes including dendritic or glial cell arborization (Spradling et al., 2011; Koo et al., 2018). In humans, brain morphometric analysis based on T1 weighted magnetic resonance imaging (MRI) scans of GW veterans exposed to the chemical weapon sarin showed overall reductions in grey matter (GM) and selective reductions in hippocampal subfield volumes when compared with unexposed veterans (Chao et al., 2011, 2015). In the white matter (WM), an overall reduction in tissue volume was observed in a dose–response manner in GW veterans with air plume-modeled exposure to sarin (Heaton et al., 2007). These WM volumetric changes in sarin exposed veterans have also been validated in other cohorts and correlated with cognitive outcomes (Proctor et al., 2006; Chao et al., 2010). More recently, two investigations using diffusion tensor imaging (DTI) have reported altered brain connectivity which correlated with fatigue, pain, or hyperalgesia in GW veterans with sarin exposure and in those with GWI (Rayhan et al., 2013; Chao et al., 2015). In both studies, enhanced axial diffusivity in the major WM tract pathways was suggested as a potential biomarker for GWI and was associated with more severe health symptom reporting (Rayhan et al., 2013; Chao et al., 2015). These findings indicate a structure–-function relationship between WM changes and chronic health symptoms in GW veterans that may be related to chronic microglial activation and neuroinflammatory cytokine signaling from damaged neural cells including more subtle neurite microstructural alterations signaling to nearby glia (O'Callaghan et al., 2015; Banks and Lein, 2012; Rathbone et al., 2015).

Mild traumatic brain injury (mTBI) is another factor that can produce a secondary neuroinflammatory response post-injury (Kumar and Loane; 2012, Rathbone et al., 2015). mTBI is the most common type of traumatic brain injury affecting military personnel. More than 15 percent of returning members experienced mTBI (Hoge et al., 2008) and it has recently been shown to be highly prevalent (~30%) in the large, longitudinally-followed Ft. Devens cohort of GW veterans and in the Boston Gulf War Illness Consortium (GWIC) cohort of GW veterans (Hoge et al., 2008; Yee et al., 2016; Janulewicz et al., 2018). Increasing evidence suggests that a single mTBI may produce long-term progressive damage in GM and WM, and accelerate age-related neurodegeneration and neuroinflammatory signaling (Bramlett and Dietrich, 2002; Smith et al., 2013; Rathbone et al., 2015; Chao, 2018). In addition, it has recently been shown that GW veterans with a mTBI history alone or in addition to sarin chemical weapons (CBW) exposure during the war are more likely to report persistent and debilitating chronic health symptoms and medical conditions suggesting that multiple mTBIs or a single mTBI and chemical weapons exposure act as multiple-hits to the neuroimmune system that primes stronger and longer neuroinflammatory signaling in those exposed (Yee et al., 2017; Janulewicz et al., 2018; O'Callaghan and Miller 2019). However, brain imaging outcomes in GW veterans with mTBI and with chemical weapons exposures during the war and their effect on microscale morphological changes including dendritic or glial cell arborization and neuroinflammatory signaling have yet to be reported.

We have previously demonstrated that high-order diffusion MRI showed a sensitivity to discriminate different stages of neuroinflammatory signaling in our established, OP exposed GWI animal model utilizing combined exposure to exogenous corticosterone at levels mimicking high physiological stress and the sarin surrogate, DFP (Koo et al., 2018). When combined with findings from other similar animal model studies, results suggest a strong brain-immune component to GWI that could be measured through brain imaging and peripheral blood immune markers and validated in GW veteran cohorts (O'Callaghan et al., 2015, Spradling et al., 2011).

Neurite density imaging (NDI, Zhang et al., 2012) and Q-space imaging (Yeh et al., 2010) are two novel diffusion processing methods of the high-order diffusion MRI measures that have been shown to successfully detect local microscale diffusivity of axon and dendrite processes in animals and human studies of neurological disorders (Colgan et al., 2016, Zhang et al., 2012, Koo et al., 2018, McCunn et al., 2019). NDI compartmentalizes the brain environment into three components to sample microstructural diffusivity, and restricted diffusion imaging measure (RDI) in Q-space imaging method provides diffusion displacement in the three-dimensional space that could provide similar diffusion information of NDI by analyzing different boundaries in the three-dimensional space (Zhang et al., 2012; Yeh et al., 2010, 2017). Both NDI and RDI can provide detailed description of microscale diffusivity of brain tissues and nearby free water space without applying predefined linear diffusion models as seen in conventional DTI approaches (Tuch, 2004; Zhang et al., 2012). Decomposing slow diffusion components with links to subneuronal, glial or extracellular compartments may give detailed insights on pathophysiologic profile of disease.

In this study, we investigate whether an NDI processing model of high-order diffusion MRI can successfully identify and validate the different levels of microstructural and macrostructural brain alterations previously seen in animal models of GWI by utilizing RDI (Koo et al., 2018) and assessing how these patterns overlap in veterans with GWI from the Boston Gulf War Illness Consortium. We also assessed the relationship between brain imaging measures, blood neuroinflammatory markers, and self-reported health symptoms in veterans with GWI and GW control veterans. Lastly, we compared the separate and combined effects of mTBI and chemical weapons exposure on high-order microstructural diffusion MRI, blood neuroinflammatory markers, and health symptom outcomes.

2. Materials and methods

2.1. Participants

The study population included 91 GW veterans from the Boston University Gulf War Illness Consortium (GWIC). The GWIC is a multi-site study that includes a series of preclinical and clinical studies designed to understand the pathobiological mechanisms responsible for the chronic symptoms of GWI and to identify diagnostic markers and targeted treatments for the disorder. GWIC inclusion criteria required deployment to the Persian Gulf between August 1990 and July 1991. GWIC exclusion criteria included diagnoses of chronic medical illnesses that could otherwise account for the symptoms experienced by GW veterans. These diagnoses included autoimmune, central nervous system, or major psychiatric disorders that could affect brain and immune functions (e.g., epilepsy, stroke, severe head injury, brain tumor, multiple sclerosis, Parkinson's disease, Alzheimer's disease, schizophrenia, bipolar disorder, and autoimmune disorders). Each of the study participants completed an assessment protocol including health surveys, a neuropsychological test battery, brain imaging, and collection of blood and saliva samples (Janulewicz et al., 2018). All participants provided written informed consent to participate in the study. This study was reviewed and approved by the Boston University institutional review board.

2.1.1. Gulf war illness criteria

GWI case status was defined from the Kansas GWI case definition (Steele, 2000). The Kansas GWI case definition requires GWI cases to endorse multiple or moderate-to-severe chronic symptoms in at least three of six statistically-defined symptom domains: fatigue/sleep problems, somatic pain, neurological cognitive/mood symptoms, gastrointestinal symptoms, respiratory symptoms and skin abnormalities (Steele, 2000). GWIC participants not meeting Kansas GWI or exclusionary criteria were considered controls. Veterans were excluded from being considered GWI cases, for purposes of the research study, if they reported being diagnosed by a physician with medical or psychiatric conditions that would account for their symptoms or interfere with their ability to report their symptoms.

2.1.2. Self-Reported mild traumatic brain injury (mTBI)

To determine mTBI status, participants were given a concussion definition that follows the current guidelines from the American Academy of Neurology and was used in our prior GW veteran mTBI publications (Vynorius et al., 2016; Robbins et al., 2014; Seichepine et al., 2013; Janulewicz et al., 2018; Yee et al., 2016; Yee et al., 2017). Participants were provided with the mTBI definition and examples of common symptoms associated with mTBI and were then asked to report if they had experienced mTBI during their deployment, they were also asked to self-report how many mTBIs they had experienced during the war.

2.1.3. Chemical/Biological weapon (CBW) exposure

GWIC subjects were administered the Kansas Gulf War Experiences and Exposure Questionnaire, and the Structured Neurotoxicant Assessment Checklist (SNAC) to assess for deployment-related exposures (Proctor et al., 1998; Steele 2000; Proctor et al., 2006). Self-reported exposures to chemical or biological weapons (CBWs) were obtained from the SNAC by asking the veterans whether or not they were exposed to CBWs during military service (Proctor et al., 1998).

2.1.4. Demographics and health symptom surveys

GWIC subjects were also administered a general demographic information and medical conditions questionnaire and the Kansas Gulf War and Health Questionnaire (Proctor et al., 1998; Steele 2000). Additional validated health symptom surveys were completed by study participants and included the Multidimensional Fatigue Inventory (MFI-20), McGill Pain Inventory and the Pittsburgh Sleep Quality Index (PSQI) where higher scores indicated more symptoms (Buysse et al., 1989; Smets et al., 1995; Melzack, 1975).

2.1.5. Cytokines

EDTA plasma was separated and stored at − 80 °C until assayed. Cytokines were measured with an 18-multiplex chemiluminescent assay using Quansys Q-view Imager LS 1.3 and reagents in methods previously reported (Fletcher et al., 2009). Each 18-multiplex plate was imaged at 500 sec, 270 sec, 180 sec, 120 sec. Following the manufacture's protocol, the 270-sec images were used for further analysis. All plates were normalized by using an internal plasma control (pooled plasma from 50 men and 50 women). This internal control (IC) was run on each plate, average pg/ml was calculated for IC across plates and each plate normalized to the percent change from IC average. This normalization removes variability between plates. In instances when the cytokine expression was below the level of detection (BLD), the difference between the lower limit of detection and 0 was used. To determine if circulating proinflammatory cytokines levels were different between GWI cases and controls, plasma samples were examined by symptom group. In this study, chemiluminescent imaging concentrations of three cytokines in plasma samples were examined and compared to the brain imaging measures. Cytokines of interest were Interleukin 1 alpha (IL1α), Tumor necrosis factor receptor type I (TNFRI) and Tumor necrosis factor receptor type II (TNFRII) based on previously demonstrated relationships between GWI and blood cytokine measures (Jaundoo et al., 2018; O'Callaghan et al., 2015; Khaiboullina et al., 2014; Broderick et al., 2011).

2.2. Image acquisition

All MRI scans were performed on an Achieva 3 T whole-body MRI scanner (Philips Healthcare, Best, The Netherlands) in the center of biomedical imaging, Boston university school of medicine.

2.2.1. T1 MPRAGE Acquisition: The Alzheimer's disease neuroimaging initiative (ADNI)

developed an MPRAGE sequence that was used for this study (TR = 6.8 msec, TE = 3.1 msec, flip angle = 9°, slice thickness = 1.2 mm, 170 slices, FOV = 250 mm, matrix = 256 × 256). We used the MPRAGE scan to generate the anatomical regions of interest (ROI) for assessing morphometric differences between the groups and also to provide anatomical co-registration with the DTI and fMRI data sets.

2.2.2. Diffusion MRI: The diffusion MRI data were obtained using a single-shot EPI sequence

with multi-shell diffusion encoding (b-value used = 1000, 2000, and 3000 s/mm2). We used 124 gradient directions utilizing parallel imaging on a 16-channel parallel head coil (70 slices, TR = 13214 msec, TE = 55 msec, with a matrix size of 128 × 128 yielding a resolution of 2.0 × 2.0 × 2.0 mm^3, no slice gap). In

addition to distortion corrections built into the scanner, we also collected 6 B0 field maps for further distortion correction.

2.3. Image processing and anatomical defining

2.3.1. Defining GM anatomy

Defining anatomical structures in the cortex was the first step in analyzing brain images. MPRAGE structural scans were analyzed using FreeSurfer (Fischl, 2012) to obtain measures of volume, cortical thickness and surface geometry for each anatomical ROIs implemented in the brain atlas (Desikan et al., 2006). Seventy-eight ROIs defined in the average template space were co-registered to each subject's cortical surface by applying nonlinear coregistration parameters. The results were visually inspected for artifacts or incomplete segmentation. A total

of seventy-eight cortical and subcortical ROIs were chosen for the analysis.

2.3.2. Defining WM anatomy

Diffusion MRI was registered to the structural MRI following the motion and eddy current distortion correction (Jenkinson et al., 2012). TRACULA (TRActs Constrained by UnderLying Anatomy) software was used to perform tract-based analysis on the preprocessed diffusion MRI data (Yendiki et al., 2011). Eighteen major white matter tracts were reconstructed for each subject.

2.4. High-order diffusion processing

To reconstruct microstructural information from high-order diffusion MRI, Neurite Density Imaging (NDI) processing was performed on merged high-order diffusion MRI images containing 3 different b- value encodings (Zhang et al., 2012). NDI applies a two-level approach by separating the volume fraction of Gaussian isotropic diffusion, representing the cerebrospinal fluid (CSF) water component. Then, the remaining diffusion signal is sub-compartmentalized into components from intra and extra-neurite water (Zhang et al., 2012). This modeling procedure provides a neurite density (ND) index, a fraction of tissue composed of axons or dendrites, and the fraction of tissue other than neurites. Orientation dispersion (OD) index provides the spatial configuration of the neurite structures based on the composite pattern of intra and extracellular diffusivity. Both ND and OD measures in each voxel were merged into 18 WM major tracts to extract tract-wise measures. For the GM and subcortical GM diffusivity assessment, diffusion modeling parameters were determined by iterative parameter selection methods based on the maximum likelihood estimation of modeling fitting error. These three different measures from this step were then merged into the 78 GM ROIs to extract ROI-wise NDI measures.

2.5. Statistical analysis

Group differences on ROI levels between GW veteran controls (GW Cont) and veterans with GWI (GWI Case) were assessed by generalized linear regression models controlling potential confounding variables such as age and gender (Gur et al., 1991). Significant p-values ($p < 0.05$) were first calculated through nonparametric permutation tests with 10,000 permutations (Winkler et al., 2014), then we applied the Benjamini & Hochberg procedure to control the false discovery rate (FDR) (Benjamini and Hochberg, 1995; Groppe et al., 2014). Significant p-values after permutations (p) or FDR adjustment (FDR_adj_p) in the whole GM and WM group comparisons were reported along with *t*-values.

Partial correlations controlling for age and gender were applied on: (1) Multidimensional Fatigue Inventory scale (MFI) and GM NDI data; (2) Pittsburgh Sleep Quality Index (PSQI) sleep score and GM NDI data; (3) plasma blood cytokine data and GM NDI data. Both whole group and subgroup level analyses were assessed in this study. Significant p-values after permutations (p) or FDR adjustment (FDR_adj_p) in the whole group and subgroup levels were reported along with the Pearson correlation coefficients (rho). For subgroups analyses with small sample sizes, we included 95% confidence intervals (95% CI)

3. Results

3.1. Demographic results

The first 91 GWIC veterans with brain imaging completed were the participants in this study. 75 GW veterans met Kansas criteria for GWI (GWI Cases) and 16 GW veterans did not meet Kansas GWI criteria and were considered GW veteran controls (GW Controls). Veterans with GWI were further divided into subgroups based on self-reported exposures to chemical weapons (CBW) or mTBI during their deployment. Those exposed to mTBI during deployment (GWI + mTBI; n = 23), CBW agents (GWI + CBW; n = 33) or both exposures (GWI + mTBI + CBW; n = 12) (Table 1).

Table 1
Demographic and self-reported exposure to risk factors information for GWI case and control subjects.

	GW Control	GWI Case
N	16	75
Age (years)	53.85	52.07
Gender (F/M)	1/15	16/59
Exposure to risk factors during war (% exposed)		
Mild traumatic brain injury (mTBI)	0%	30.67%
Chemical/Biological warfare agents (CBW)	12.50%	44%
mTBI + Chem/Bio warfare agents (mTBI + CBW)	0%	16%

3.2. GWI decreases NDI measures in both WM and GM regions

Whole group analysis in both WM and GM imaging measures indicated significant differences between GWI cases and controls, with p-values < 0.05 after FDR correction (Fig. 1, Sup.1, Sup.2).

Compared to controls, significantly decreased patterns in GWI cases were seen in ND for all major WM tracts. Both ND and OD showed decreased patterns for most GM ROIs. The highest significant group differences between GWI cases and controls were seen in the left cingulum angular bundle (cab, $t = -2.963$, FDR_adj_p = 0.027), the bilateral uncinate fasciculus (unc, $t = -2.749$, FDR_adj_p = 0.026 (left), $t = -2.941$, FDR_adj_p = 0.026 (right)), the bilateral rostral anterior cingulate ($t = -3.272$, FDR_adj_p = 0.026 (left), $t = -2.882$, FDR_adj_p = 0.026 (right)), and the bilateral fusiform gyrus ($t = -3.006$, FDR_adj_p = 0.026 (left), $t = -2.909$, FDR_adj_p = 0.026 (right)) (Fig. 1, Sup.1, Sup.2).

3.3. GWI subgroups have distinct patterns of behavioral symptoms and brain changes

Specific risk factors were selected to define subgroups for correlation analysis to self-reported health symptom measures. GM ND and self- reported symptom scores within mTBI, CBW and mTBI + CBW subgroups showed an overall negative relationship, but highlighted specific regions in each subgroup (Fig. 2, Sup. 3, Sup. 4). There were more localized patterns in GWI + mTBI ND and OD measures, with the most significant results seen in the left pars orbitalis for the MFI score (rho = −0.706, FDR_adj_p = 0.027, 95% CI = [−0.859, −0.389]) and the left lingual gyrus for the PSQI score (rho = −0.709, FDR_adj_p = 0.036, 95% CI = [−0.860, 0.374]) (Fig. 2, Sup. 3, Sup. 4). Conversely, the GWI + CBW subgroup has more widespread and bilateral patterns for both ND and OD, some of the most significant results seen in the bilateral rostral anterior cingulate for the MFI score (rho = −0.655, FDR_adj_p = 0.002, 95% CI = [−0.803, −0.373] (left), rho = −0.605, FDR_adj_p = 0.002, 95% CI = [−0.771, −0.297] (right)) and the bilateral caudal anterior cingulate for the PSQI score (rho = −0.520, FDR_adj_p = 0.038, 95% CI = [−0.688, −0.129] (left), rho = −0.493, FDR_adj_p = 0.038, 95% CI = [−0.779, −0.316] (right)) (Fig. 2, Sup. 3, Sup. 4). The GWI + mTBI + CBW group showed enhanced patterns in restricted regions found in the single risk factor subgroup analysis, with the most significant results seen in the bilateral caudal middle frontal gyrus (rho = −0.804, FDR_adj_p = 0.036, 95% CI = [−0.949, 0.476] (left), rho = −0.808, FDR_adj_p = 0.036, 95% CI = [−0.951, 0.491] (right)) for the MFI score and the right parahippocampal gyrus for the PSQI score (rho = −0.698, p = 0.036, 95% CI = [−0.919, −0.194]) (Fig. 2, Sup. 3, Sup. 4).

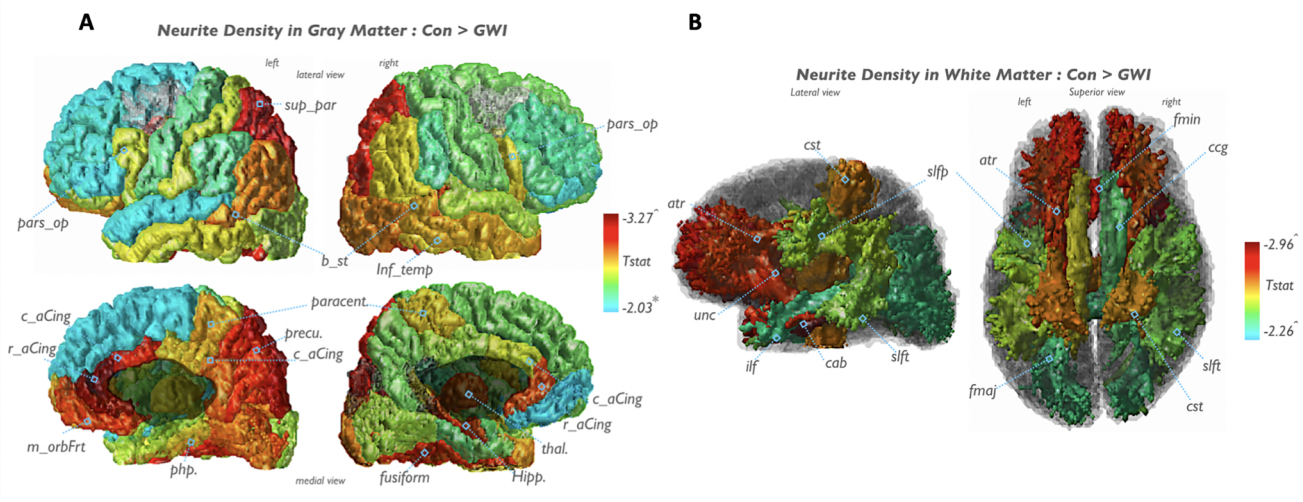

Fig. 1. ND feature mapping of whole group WM and GM analyses highlights group effects in widespread regions, most significantly seen in frontal white matter tracts and subcortical limbic regions. Fmaj = corpus callosum forceps major, fmin = corpus callosum forceps minor, atr = anterior thalamic radiations, cab = cingulum-angular bundle, ccg = cingulate gyrus bundle, cst = corticospinal tract, ilf = inferior longitudinal fasciculus, slfp = superior longitudinal fasciculus parietal, slft = superior longitudinal fasciculus temporal, unc = uncinate fasciculus, pars_op = pars opercularis, sup_par = superior parietal, b_st = banks of superior temporal sulcus, inf_temp = inferior temporal, c_aCing = caudal anterior cingulate, r_aCing = rostral anterior cingulate, m_orbFrt = medial orbitofrontal, php = parahippocampal, hipp = hippocampus, thal = thalamus proper, precu = precuneus, paracent = paracentral. * $p < 0.05$,^ FDR_adj_p < 0.05.

3.4. Peripheral immune markers are associated with decreased NDI measures

Plasma cytokine markers showed negative relationships with NDI measures within the subgroups (Fig. 3, Sup. 5, Sup. 6, Sup. 7). Specifically, in the GWI + mTBI group, TNFRI and TNFRII showed significant negative correlations with the left entorhinal cortex (TNF RI: rho = −0.439, p = 0.041, 95% CI = [−0.707, −0.006]; TNF RII: rho = −0.523, p = 0.015, 95% CI = [−0.758, −0.115]) and the left parahippocampal gyrus (TNF RII: rho = −0.461, p = 0.036, 95% CI = [−0.735, −0.063]) regions (Sup. 5, Sup. 7). Additionally, partial correlation analysis of IL1A revealed the most significant relationship with the left middle temporal gyrus (rho = −0.567, p = 0.008, 95% CI = [−0.804, −0.229]) (Sup. 5, Sup. 7). In the GWI + CBW group, TNFRII had significant negative correlations with many bilateral cortices including the entorhinal, cingulate, parahippocampal, thalamus, occipital and temporal regions. The bilateral entorhinal cortices had the most significant negative correlation to TNFRII (rho = −0.525, p = 0.002, 95% CI = [−0.721, −0.192] (left), rho = −0.418, p = 0.017, 95% CI = [−0.669, −0.093] (right)) (Fig. 3, Sup. 5, Sup. 6).

4. Discussion

This study showed that the NDI model of high-order diffusion MRI processing detected detailed microstructural alterations in WM tracts and GM ROIs in veterans with GWI, which validated results from our previous work utilizing the GWI rat model where neuroinflammation, as measured by increased brain cytokine signaling, was correlated with high-order diffusion MRI in toxicant-exposed animals (Koo et al., 2018). Our major findings are 1) Veterans with GWI showed widespread microstructural changes compared to control veterans in both ND and OD measures, with the most pronounced differences in the frontal white matter tracts and the limbic/paralimbic cortical regions, 2) Veterans with more pronounced brain changes reported higher rates of exposure to mTBI and CBW during their deployment, 3) Veterans with CBW exposure showed widespread microstructural brain changes while those with mTBI showed more focal microstructural changes on high-order diffusion MRI. 4) Behavioral symptoms were associated with distinct brain changes across the GWI exposure subgroups, and 5) Peripheral immune cytokine markers correlated with increased fatigue and sleep symptoms and with brain NDI measures in veterans with GWI indicating structure–function relationships between brain imaging, inflammatory markers, and behavioral outcomes.

The tissue water diffusion information captured in diffusion MRI can be potentially sensitive to many factors including axons, dendrites as well as myelinated fibers, changes in the neuroglial cells may also be a potential factor for differential patterns in water diffusivity (Gulani et al., 2001; Naughton et al., 2018; Belgrad et al., 2019). Water diffusivity may differ from either loss of existing neurons or reproduced neurons (neurogenesis) in the tissue medium. Also, changes in morphology in neuroglial cells take place during different stages of activation thereby resulting in differential patterns of water diffusivity in the brain (Raivich et al., 1999). Considering all these components, variations in the tissue environment might be expressed in a mixture of diverse diffusion strengths. A significant loss in cell populations can impact fast (i.e., macroscopic) water diffusion components since there will be less barriers for restricting water diffusion in the cell medium (Johnson et al., 2014). On the other hand, changes in sub-neuronal components, such as synaptogenesis or glial activation, can increase complexity in the medium and thereby change distinct diffusion components compared to the neuronal loss (Zhuo et al., 2012). While DTI measures could provide overall information of microstructural tissue changes in the brain, common markers of DTI, mean diffusivity (MD) and fractional anisotropy (FA), take in account of changes in all tissue components, hence novel approaches such as NDI and RDI could provide more specific information on the aforementioned changes in different tissue components as well as fiber orientation estimation (Tuch, 2004; Zhang et al., 2012)

OP nerve agents induce neuroinflammatory responses in cortical structures including limbic and paralimbic structures (Spradling et al., 2011; Rao et al., 2017; Naughton et al., 2018). Such neuroinflammatory responses might result from neurological damage as a result of neurotoxicant exposure and damage signaling to innate immune cells (Milligan and Watkins, 2009). However, the level of damage might also show mild long-lasting changes in sub-neuronal components and morphometry of neurite cells including axons and dendrites rather than the remarkable loss of neurons (Spradling et al., 2011; O'Callaghan et al., 2015). The lower range of diffusion encodings used in diffusion MRI (typically, around b = 1000 s/mm^2) is the most common protocol in

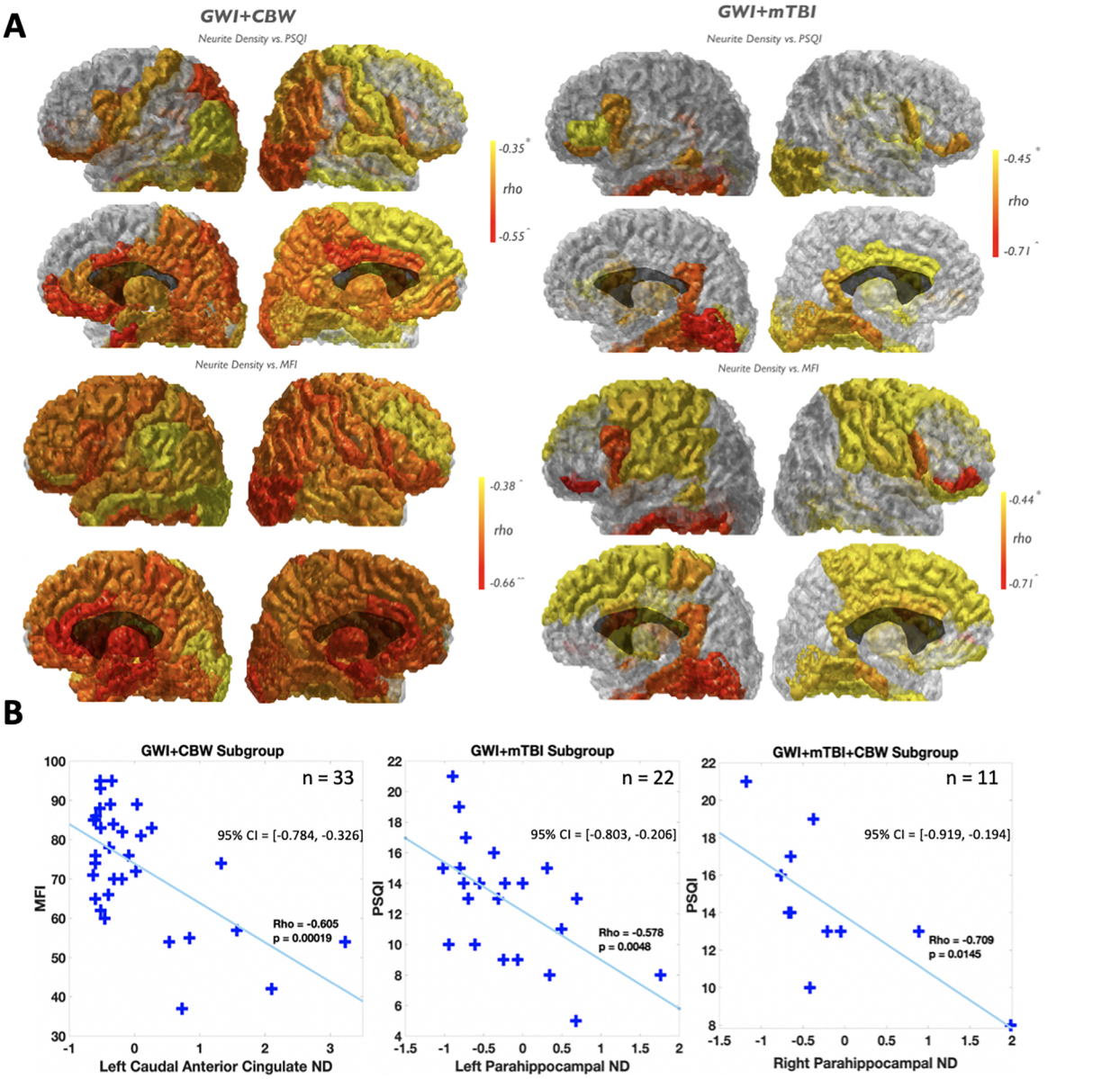

Fig. 2. Self-reported symptoms correlation mapping in GWI subjects exposed to chemical or biological warfare agents or mTBI. Regions with significant correlation between ND and PSQI (A, left upper) or MFI (A, left lower) in GW veterans with chemical/biological warfare agent exposures are rendered based on significance levels. Regions with significant correlation between ND and PSQI (A, right upper) or MFI (A, right lower) in GW veterans with mTBI exposure are rendered based on significance levels. Panel B shows data distribution patterns of ND and PSQI (B, middle and right) or MFI (B, left) scores in representative regions within each subgroup. Some subjects did not have available PSQI data, therefore, the number of subjects (n) used for subgroup correlation is indicated in the figure and 95% CIs are provided. * $p < 0.05$,^ FDR_adj_p < 0.05,^ FDR_adj_p < 0.01.

clinical imaging. Under this protocol, diffusion MRI has been a powerful tool for assessing WM major pathways, edema, or brain tumors. However, it does not have enough sensitivity to assess the mild progressive damage in the sub-neuronal components since the sub-neuronal component alterations including axonal microtubule density and stability changes, myelin depletion and oligodendrocyte function and arborization of dendrites or glial process morphometry changes might induce changes in variant forms of microscopic water diffusivities (Rao et al., 2017; Naughton et al., 2018; Belgrad et al., 2019). In our previous study on GWI animal model brain imaging, we confirmed neurotoxicant-induced neuroinflammatory response accompanies microscale changes in the neuronal cell environment that significantly correlated with proinflammatory cytokine signaling (Koo et al., 2018). These results also highlight the ability to detect inflammatory-induced changes in microstructural diffusion imaging. The results from our previous work were the rationale for studying separate diffusion components on brain imaging in GW veterans with various exposures and peripheral cytokine markers.

Based on the high-order diffusion MRI, we have confirmed that the NDI successfully and significantly differentiated between veterans with and without GWI. While NDI measures revealed overall and widespread pattern differences between groups, the clearest distinctive pattern was confirmed in the limbic/paralimbic structures along with the anterior WM connections. However, little significant differences were observed in DTI measures in major WM tracts (Sup. 11). In addition to WM, GM diffusion mapping provided a clear explanation of the relationship between microstructural damage and illness symptoms. Considering the cytoarchitectural profiles of the cortical structures, GM measures from high- order diffusion MRI may reflect distinct patterns of microstructural damage across regions. As previously discussed (Glasser

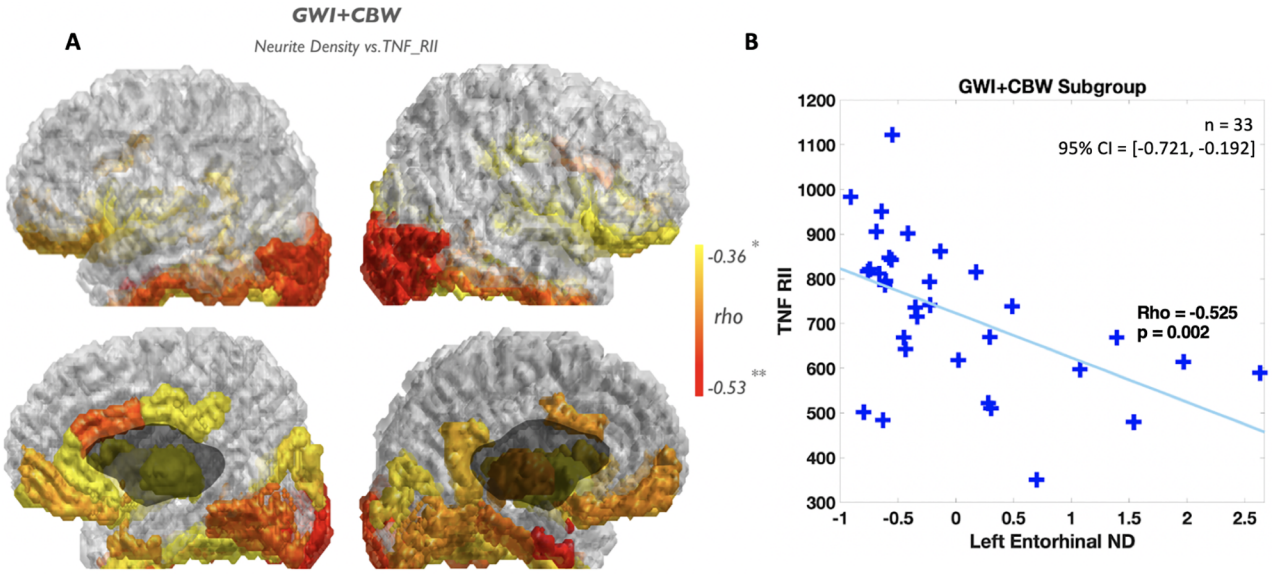

Fig. 3. Blood cytokine correlation mapping in GWI subjects exposed to chemical and biological warfare agents. Regions with significant correlation between ND and TNF_RII (A) are rendered based on significance levels. Panel B shows data distribution patterns of ND and TNF_RII levels in the representative region within the GWI + CBW subgroup. * $p < 0.05$, ** $p < 0.01$.

et al., 2014), neuronal density in brain regions co-varies with myelinated axons. While NDI could be sensitive to myelinated axons (Fukutomi et al., 2018; Grussu et al., 2017), lowered ND in both medial prefrontal regions and anterior WM tracts may reflect damage in myelinated axons. However, other regions had more dominant changes in GM than in the WM. The cingulate cortex and parahippocampal area have relatively thick cortical layers and unmyelinated fibers. These regions may account for different neurological sources for NDI mapping. Similar to what we have confirmed from the animal model of GWI using RDI measure (Koo et al., 2018), we have found a strong link between NDI measures and RDI measure on the GW human data used in this study, suggesting NDI profiles may also account for neuroinflammatory responses in the brain (Sup. 11). Indeed, some of our NDI mappings, such as the precuneus and the anterior cingulate cortex, have overlapped patterns to those of a recent GWI study using the translocator protein (TSPO) based positron emission tomography imaging (Alshelh et al., 2020). This may indicate that NDI contrasts can be affected by activated glial cell populations in local brain regions.

Multiple risk factors have been investigated in search of the underlying causes of GWI symptoms, suggesting a neuroinflammatory etiology due to individual or multiple neurotoxicant exposures during deployment (White et al., 2016; Abou-donia et al., 2017; Sullivan et al., 2017). Recent studies have identified mTBI to play a significant role in increased rates of health-related symptoms (Yee et al., 2016; Yee et al., 2017, Chao, 2018; Janulewicz et al., 2018) in GW veterans whereas OP chemical warfare agents were critical risk factors to GWI symptoms specifically (Chao et al., 2010, 2011, 2015). Besides, high-order diffusion MRI has previously been shown to detect microstructural changes in a rat model of mTBI (Zhuo et al., 2012). As a result, we focused on GWI cases with either one or both of those risk factors as separate subgroups for further analysis and to recapitulate existing results. In this study, mTBI groups showed more focal diffusion changes while the CBW exposed group showed more widespread diffusion changes in the WM tracts and the GW ROIs. Similar to what we confirmed with GWI animal models, this may indicate that microscale changes in the neuronal cell environment can be a potential biomarker for explaining illness symptoms in GWI and groups with specific brain insults (physical and chemical) during the war (Koo et al., 2018). However, further testing in a large scale sample is needed to draw integrative and generalizable conclusions.

4.1. Behavioral symptoms and associated brain changes

Due to the complex, multi-symptomatic etiology of GWI, various clinical and self-reported symptom measures were used in our correlation analysis to investigate the relationship between imaging results and symptom severity. Overall, subjects with more depleted ND and OD reported worse sleep quality on PSQI and higher fatigue levels on the MFI indicating objective markers for subjective symptom complaints. We observed the most significant correlation between imaging data and MFI scores indicated a strong CNS component to fatigue in GWI. Fatigue symptoms showed strong associations with decreased parahippocampal measures, which is consistent with previous studies on GM volumes in other disorders including chronic fatigue syndrome (Puri et al., 2012; Tang et al., 2015; Kimura et al., 2019). Limbic and nearby related paralimbic areas had the most altered GM integrity and also displayed the most significant negative relationships among all regions in addition to the particular regions responsible for each symptom.

4.2. TNF mediated inflammation

Proinflammatory cytokine levels in the blood could be used as markers to indirectly analyze CNS innate immune responses after exposures or experiences to noxious external stimuli, which in GWI studies were often chemical warfare agents and exposures to similar classes of chemicals (Michalovicz et al., 2019). Exposure to neurotoxicants such as sarin, PB, pesticides, and other chemical warfare agents has been identified to pose negative health effects in GW veterans in cohort studies (Chao et al., 2010, 2011,2015; Sullivan et al., 2003; Sullivan et al., 2017; Zundel et al., 2019) and controlled animal studies (Abdullah et al., 2011). Indeed, the GWI + CBW group displayed significantly upregulated TNF RI and TNF RII along with decreased ND in frontal and subcortical limbic regions, similar regions highlighted with symptom-specific domains. The main ligand for both TNF RI and TNF RII, TNFα is a potent inflammatory cytokine released by macrophages triggering numerous events including apoptosis, edema, and leukocyte adhesion (Zelová and Hošek, 2013). Receptor shedding has been proposed as a mechanism to counteract high levels of TNFα to balance inflammatory responses (Xanthoulea et al., 2004; Hawari et al., 2004). Previous studies have shown TNFα to be a significant biomarker for

GWI (Broderick et al., 2011; Khaiboullina et al., 2014; O'Callaghan et al., 2015; Jaundoo et al., 2018). However, unlike what we confirmed from TNF RI and RII, we did not see significant patterns in TNFα in this study. The discrepancy between these measures should be determined in further studies to clarify the role of the TNF pathway in mediating inflammation, which may contribute to the fatigue and sleep symptoms of the disease.

5. Conclusion

Our study provides neuroimaging evidence underlying GWI etiologies and reveals GWI-specific microstructural changes in the frontal and subcortical paralimbic regions due to mTBI and chemical weapons exposures. We showed for the first time in GW veterans that mTBI was associated with discrete focal microstructural changes on MRI and that chemical weapons exposures resulted in more diffuse and widespread microstructural changes on brain imaging. In addition, these microstructural brain changes correlated with peripheral neuroinflammatory markers in the blood of veterans with GWI. When these results are combined with our prior studies showing correlations with brain cytokines and microstructural changes in the GWI animal model, this provides compelling evidence for neuroinflammation in the pathobiology of GWI. This is especially the case given that the NDI microstructural brain changes also negatively correlated with the self-reported markers of fatigue and sleep on the MFI and PSQI which suggests functional consequences from these structural changes and also validates their use as objective measures and validating NDI imaging as a potential marker of treatment trial efficacy pre- and post-treatment for GWI symptoms. Correspondingly, current GWI literature on microstructural alterations due to neuroinflammation in the limbic areas have indicated changes in memory and emotion-related functions as evidenced by psychological and health outcome correlational studies (Toomey et al., 2009; Chao et al., 2010; Abdullah et al., 2011; Chao et al. 2011; Sullivan et al., 2003; Janulewicz et al., 2018; Sullivan et al., 2017; Jeffrey et al., 2019). However, there are several limitations to human studies, which can be overcome with concurrent controlled animal experiments as we have done in our ongoing GWIC studies (O'Callaghan et al., 2015; Koo et al., 2018). Further studies are needed to elucidate which neuronal and glial changes are contributing to diffusion imaging results seen here and how microstructural alterations may lead to higher risks of accelerated aging and earlier risks for neurodegenerative and cerebrovascular diseases in GW veterans so that intervention strategies can be implemented (Barnes et al., 2018; Smith et al., 2013; Zundel et al., 2019).

Acknowledgements

Ms. Cheng and Dr. Koo contributed equally to this work as the 1st author. Drs. Koo and Sullivan are the corresponding authors. This work is supported by a department of Defense CDMRP new investigator award (W81XWH-17-1-0440) and CDMRP GWI consortium award (W81XWH-13-2-0072).

Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention. This work was supported by the Assistant Secretary of Defense for Health Affairs, through the Gulf War Illness Research Program, GW160032 and GW120037. Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the Department of Defense.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2020.07.006.

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Host *Akkermansia muciniphila* Abundance Correlates With Gulf War Illness Symptom Persistence via NLRP3-Mediated Neuroinflammation and Decreased Brain-Derived Neurotrophic Factor

Neuroscience Insights
Volume 15: 1–15

DOI: 10.1177/2633105520942480

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ABSTRACT: Neurological disorders are commonly reported among veterans who returned from the Gulf war. Veterans who suffer from Gulf War illness (GWI) complain of continued symptom persistence that includes neurological disorders, muscle weakness, headaches, and memory loss, that developed during or shortly after the war. Our recent research showed that chemical exposure associated microbial dysbiosis accompanied by a leaky gut connected the pathologies in the intestine, liver, and brain. However, the mechanisms that caused the symptoms to persist even 30 years after the war remained elusive to investigators. In this study, we used a rodent model of GWI to investigate the persistence of microbiome alterations, resultant chronic inflammation, and its effect on neurotrophic and synaptic plasticity marker BDNF. The results showed that exposure to GW chemicals (the pesticide permethrin and prophylactic drug pyridostigmine bromide) resulted in persistent pathology characterized by the low relative abundance of the probiotic bacteria *Akkermansia muciniphila* in the gut, which correlated with high circulatory HMGB1 levels, blood-brain barrier dysfunction, neuroinflammation and lowered neurotrophin BDNF levels. Mechanistically, we used mice lacking the NLRP3 gene to investigate this inflammasome's role in observed pathology. These mice had significantly decreased inflammation and a subsequent increase in BDNF in the frontal cortex. This suggests that a persistently low species abundance of *Akkermansia muciniphila* and associated chronic inflammation due to inflammasome activation might be playing a significant role in contributing to chronic neurological problems in GWI. A therapeutic approach with various small molecules that can target both the restoration of a healthy microbiome and decreasing inflammasome activation might have better outcomes in treating GWI symptom persistence.

KEYWORDS: Dysbiosis, *Akkermansia muciniphila*, BDNF, RAGE, 3-nitrotyrosine, peroxynitrite, inflammasomes, NLRP3

RECEIVED: May 31, 2020. **ACCEPTED:** June 25, 2020.

TYPE: Gulf War Illness (GWI) and Nervous System Disorders - Original Research

FUNDING: The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Funding for this project was provided by DoD-IIRFA grant: W81XWH1810374 and VA merit award I01 CX001923-01 to Saurabh Chatterjee; W81XWH-16-1-0556 to Dr Stephen Lasley; W81XWH-13-2-0072 to Dr Kim Sullivan. This material is based upon work supported (or supported in part) by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development vide grant no. CX001923-01 to Saurabh Chatterjee. Chatterjee is a 5/8th employee with the US Department of Veterans Affairs.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Introduction

Neurological disorders are commonly reported among veterans who returned from the Gulf war (GW) of 1990-1991.[1-3] Afflicted veterans complain of problems including neuralgias, migraine headaches, muscle weakness and coordination, and memory problems.[2,4,5] These issues occur in combination with other Gulf War illness (GWI) symptoms, and their pathology is not very well understood. Most veterans who suffer from GWI developed their symptoms during or shortly after the war, and these symptoms persist 30 years later. Although the causes of these symptoms are difficult to pinpoint, epidemiological studies have established a compelling link between these symptoms in different GW veteran cohorts and environmental exposures which occurred during the war, or chemicals that were applied to the warriors before or shortly after the war.[6-8] Such exposures include dust from desert storms, depleted uranium, combustion byproducts from oil wells, possible chemical weapons, pesticides, vaccines, and prophylactic medicines such as pyridostigmine bromide (PB).[9-12]

In recent years, research has focused on studying symptoms as well as elucidating mechanisms of these disorders, using GW veteran cohorts as well as animals and in vitro studies. For example, Van Riper et al[13] reported widespread disruption in white matter microstructure distribution across brain regions involved in the processing and modulating chronic pain. James et al found that there was a significant positive correlation between C-reactive protein (CRP), pain, and neurocognitive mood in GW veterans. Another study by Abou-Donia et al[14] reported

*Diana Kimono and Dipro Bose have equal contribution.

elevated autoantibodies to neurons and other brain cells, eg, tau proteins, glial acidic fibrillary protein (GFAP), and myosin basic protein (MBP) which indicate neuronal injury or gliosis in GW veterans. In other recent animal studies, Zakirova et al[15] found cognitive deficits in mice several weeks after treatment with GW chemicals, and these deficits were associated with increased astrogliosis and a reduction in synaptophysin in mouse hippocampi and cerebral cortex. Furthermore, Madhu et al[16] found that cognitive impairments which persisted 10 months post-exposure to GW chemicals were associated with increased density of activated microglia and astrocytes in rats and inflammation with elevated levels of HMGB1 in the cerebral cortex.

These studies provide evidence that exposure to GW chemicals plays a significant role in the persistence of neurological dysfunction. This may generally be through the disruption of neuronal networks, reactive glia, which fuel inflammation or weak neuronal growth and neuroplasticity. Many neurological disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), bipolar disorders, and neuropathic pain[17-21] are associated with decreased levels of neurotrophins or impairments in their signaling pathways.[22,23] These disorders also commonly present with chronic neuroinflammation.[24-26] Brain-derived neurotropic factor (BDNF) is the most prevalent neurotrophins in the brain and has been very widely studied in several diseases and brain functions.[23,27] It is produced by neurons, and it plays crucial functions in neuroplasticity, growth, and survival of neurons.[28]

To date, our previous research has focused mainly on the possible role of an altered microbiome (bacteriome and virome) in contributing to a persistent inflammatory phenotype, in acute models of GWI.[29-32] We proposed that exposure to GW chemicals alters the microbiome in rodents, which then drives inflammation through the production of immunostimulatory particles, ie, damage-associated molecular pattern (DAMPS) and pathogen-associated molecular patterns (PAMPS). These DAMPS may then continuously trigger inflammation in different organ systems. Although our results largely supported our hypothesis, we were still limited in knowing whether the observed changes and mechanisms in the microbiome and associated chronic inflammation due to an altered microbiome indeed persisted.

In this present study, we used a persistence rodent model of GWI in which mice were exposed to GW chemicals for 2 weeks (representing the war phase) after which no further GW chemicals were applied for the next 20 weeks (to represent 20 years after the war). We then investigated the persistence of microbiome alterations, chronic inflammation, and its effect on neuronal health (BDNF levels). We further used an NLRP3 KO mouse to study its potential role of this inflammasome as a primary contributor to the observed neuroinflammation.

Materials and Methods

Materials

We purchased PB and permethrin from Sigma-Aldrich (St. Louis, MO). Anti-RAGE, anti-Claudin 5, anti-HMGB1, anti-IL-1β, and anti-ASC-2 were purchased from Santacruz Biotechnology (Dallas, TX), Anti-BDNF from cell signaling technology (Danvers, MA), while anti-NLRP3, anti-3-nitrotyrosine, anti-IL-6, anti-IL-18, anti-TMEM 119 primary antibodies were purchased from Abcam (Cambridge, MA). Species specific biotinylated conjugated secondary antibodies and Streptavidin-HRP (Vectastain Elite ABC kit) were purchased from Vector Laboratories (Burlingame, CA). Fluorescence conjugated (Alexa Fluor) secondary antibodies, ProLong Diamond antifade mounting media with DAPI and Pierce LAL chromogenic endotoxin quantitation kit were bought from Thermo Fisher Scientific (Waltham, MA) while enzyme-linked immunosorbent assay (ELISA) kits were purchased from ProteinTech (Rosemond, IL). Unless otherwise specified, all other chemicals used were purchased from Sigma. Paraffin-embedding of tissue and sectioning were done by AML laboratories (Baltimore, MD) and at the Instrument Resources Facility, University of South Carolina School of medicine (Columbia, SC). Microbiome analysis was done by Cosmos ID (Rockville, MD).

Animal experiments

Adult (10 weeks old) wild type male (C57BL/6J mice) and NLRP3 deficient adult (10 weeks) male (B6N.129-Nlrp3tm3Hhf/J) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice experiments were implemented in accordance with National Institutes of Health (NIH) guidelines for humane care and use of laboratory animals and local Institutional Animal Care and Use Committee (IACUC) standards. All procedures were approved by the University of South Carolina at Columbia, SC. Mice were housed individually and fed on a chow diet at 22°C to 24°C with a 12 h light/12 h dark cycle. All mice were sacrificed after animal experiments had been completed. Right after anesthesia, blood from the mice was drawn using cardiac puncture, to preserve serum for further experimentation. Their brains were removed immediately, and the frontal cortex dissected out and was fixed using Bouin's fixative solution. We also collected the fecal pellets and luminal contents for microbiome analysis.

Treatments and rodent model of Gulf War illness

Mice were exposed to GW chemicals (PB and permethrin) based on established rodent models of GWI with some modifications.[31,33-35] The treated mice group (GWP) and NLRP3KO (GWP-NLRP3KO) mice group were dosed tri-weekly for 2 weeks with PB (2 mg/kg) and permethrin (200 mg/kg) by oral gavage, after which no further treatments with GW chemicals were applied, and mice were fed on a normal chow diet for 20 weeks. The control group (CONT) of mice received vehicle (0.6% dimethyl sulfoxide in phosphate-buffered saline [PBS]) by oral gavage as in other experiments above. Each group had a starting sample size of n = 6 until the end of the experiments. All experimental mice had an average starting weight of

26.5 grams and a final weight of 33.5 grams at the end of the study. There was no signifcant difference of weight difference between controls and Gulf War chemical treated groups over the 20 week period.

Microbiome analysis

Microbiome analysis was done by CosmosID (Rockville, MD, USA) from fecal pellets and luminal contents, which were collected from the animals of each group after sacrifice. DNA isolation, sequencing, and analysis of gut microbiome were performed according to vendor optimized protocol. Briefly, DNA was isolated from fecal samples using the ZymoBIOMICS Miniprep kit, following the manufacturer's instructions. 16S sequencing was carried out on the V3V4 (341 nt–805 nt) region of the 16S ribosomal RNA (rRNA) gene with a 2-step polymerase chain reaction (PCR) strategy. First, PCR was performed using 16S-optimized primer set to amplify the V3–V4 regions of 16S ribosomal DNA (rDNA) within the metagenomic DNA. Then the PCR products from the previous steps were mixed at equal proportions and used as templates in the second step to produce Illumina dual-index libraries for sequencing, with both adapters containing an 8bp index allowing for multiplexing. The dual-indexed library amplification products are purified using Ampure beads (Beckman Coulter). Library quantification was performed using Qubit dsDNA HS assay (Thermo Fisher) and qualified on a 2100 Bioanalyzer instrument (Agilent) to show a distribution with a peak in the expected range. A final qualitative PCR (qPCR) quantification was performed before loading onto a MiSeq (Illumina) sequencer for PE250 (v2 chemistry). The sequences for each sample were then run on the 16S pipeline of the CosmosID GENIUS software, and results were analyzed.

Laboratory Methods

Immunohistochemistry

The fixed brain tissues were embedded in paraffin and sliced into 5 µM thick sections. These sections were deparaffinized following optimized standard protocols. Epitope retrieval solution and steamer (IHC-Word, Woodstock, MD) were used for epitope retrieval for deparaffinized sections. About 3% H_2O_2 was used for the recommended time to block the endogenous peroxidase. After serum blocking, the primary antibodies were applied at recommended and optimized concentrations. Species-specific biotinylated conjugated secondary antibodies and streptavidin conjugated with HRP were used to implement antigen-specific immunohistochemistry. 3,3'-Diaminobenzidine (DAB) (Sigma Aldrich, St Louis, MD) was used as a chromogenic substrate. Mayer's hematoxylin solution (Sigma Aldrich) was used as a counterstain. Sections were washed between steps using PBS 1×. Finally, stained sections were mounted in Simpo-mount (GBI Laboratories, Mukilteo, WA). Tissue sections were observed using Olympus BX63 microscope (Olympus, America). Cellsens software from Olympus America (Center Valley, PA) was used for morphometric analysis of images.

Table 1. Primer sequences.

PRIMER	SEQUENCE
mm-Claudin 5	*Sense*: TTCGCCAACATTGTCGTCC *Antisense*: TCTTCTTGTCGTAGTCGCCG
mm-18 S	*Sense*: TTCGAACGAACGTCTGCCCTATCAA *Antisense*: ATGGTAGGCACGGCGATA

Immunofluorescence staining

Paraffin-embedded sections were deparaffinized using the standard protocol. Epitope retrieval solution and steamer were used for epitope retrieval of sections. Primary antibodies were used at recommended dilutions. Species-specific secondary antibodies conjugated with Alexa Fluor (633-red and 488-green) were used at advised dilution. In the end, the stained sections were mounted using prolong diamond antifade reagent with DAPI. Sections were observed under Olympus fluorescence microscope BX63 using 20X, 40X, 60X objective lens.

Real-time quantitative PCR

Total RNA was isolated from frontal cortex tissue homogenization in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and purified with the use of RNeasy mini kit columns (Qiagen, Valencia, CA, USA). Complementary DNA (cDNA) was synthesized from purified RNA (1 µg) using iScript cDNA synthesis kit (Bio-rad, Hercules, CA, USA) following the manufacturer's standard protocol. Real-time qPCR (qRTPCR) was performed with the gene-specific primers using Sso Advanced SYBR Green Supermix and CFX96 thermal cycler (Bio-rad, Hercules, CA, USA). Threshold cycle (Ct) values for the selected genes were normalized against respective samples internal control 18S. Each reaction was carried out in triplicates for each gene and for each sample. The relative fold change was calculated by the 2–ΔΔCt method. The sequences for the primers used for real-time PCR are provided in Table 1.

Endotoxin level detection by Litmus Amebocyte Lysate assay

Serum bacterial endotoxin levels (EU/mL) were detected using the Pierce LAL Chromogenic Endotoxin Quantification Kit (Waltham, MA) according to the manufacturer's instructions. Briefly, serum samples were obtained from mice and diluted 1:80 with endotoxin-free water. The endotoxins were then quantified.

Western blot analysis

About 30 mg of tissue from each brain tissue sample was immediately homogenized in 300 µL of RIPA buffer with protease and phosphatase inhibitors cocktail (Pierce, Rockford, IL) using slow speed mechanical homogenizer. The homogenate was centrifuged, and the supernatant was collected and saved for

experimental use. About 30 µg of denatured protein from each sample was loaded per well of Novex 4% to 12% bis-tris gradient gel (Life Technologies, Carlsbad, CA) and subjected for standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated protein bands were transferred to nitrocellulose membrane using precut nitrocellulose/filter paper sandwiches (Bio-Rad, Hercules, CA) and Trans-Blot Turbo transfer system (Bio-Rad) using 30-minute transfer protocol. Furthermore, blots were blocked with 3% bovine serum albumin solution prepared in Tris-buffered saline with 0.05% tween-20 (TBS-T). Primary antibodies were used at recommended dilutions in 1.5% blocking buffer and incubated overnight at 4°C. Species-specific anti-IgG secondary antibody conjugated with HRP were used at recommended dilutions in 1% blocking buffer and incubated for 2 h at room temperature. Pierce ECL Western Blotting substrate (Thermo Fisher Scientific Inc, Rockford, IL) was used in dark to develop the blot. Finally, the blot was imaged using G:Box Chemi XX6 (Syngene imaging systems) and subjected to densitometry analysis using Image J software.

ELISA

Serum IL-1β, IL-6, and TNF-α were estimated using ELISA kits from ProteinTech (Rosemont, IL) according to manufacturer protocol.

Statistical analysis

We conducted calculations for each experimental condition prior to initiation of the study. Preliminary data confirmed that the sample size was enough to achieve a minimum statistical power of 0.80 at an alpha of 0.05. One-way analysis of variance (ANOVA) was used with post hoc comparisons among different exposure conditions or treatments (eg, least significant differences [LSD] and Bonferroni correction) to compare means among multiple groups. Student *t* tests was used to compare means between two groups at the termination of treatment. Correlative associations were tested using Pearson's correlation coefficient analysis with Graph pad prism software (GraphPad Software Inc, La Jolla, CA). A p value of less than p=0.05 was considered statistically significant.

Results

Gulf War chemical exposure results in a decreased relative abundance of Akkermansia muciniphila, which negatively correlates with increased circulatory HMGB1 levels

Our previous studies have strongly suggested that exposure to GW chemicals alters the microbiome and these alterations may contribute to the persistence of GWI symptoms through the release of DAMPs and PAMPs.[30-32] In this study, we analyzed the microbiome for alterations in specific bacterial species that have a notable role in inflammation persistence in chronic diseases of the gut, metabolic reprogramming, and neuronal deficiencies. We analyzed 10 distinct bacterial species that had a fold change difference in abundance and has been found to contribute to inflammation and metabolic responses (Figure 1A) We found that mice treated with GW chemicals (GWP) had a significantly lower abundance of *A muciniphila* (significant, Figure 1B, $^*P = .008$; n = 5), *Bacteroides thetaiotomicron*, and *Dorea Sp* (not significant) when compared with mice treated with only vehicle control (Figure 1A). Notably, *A muciniphila* has been associated with several health benefits.[36-38] Furthermore, we found that mice which were treated with GW chemicals (GWP) had significantly higher HMGB1 levels in their serum compared with mice treated with vehicle control only (CONT) $^*P = .034$; n = 6 (Figure 1C and D). We then carried out statistical analyses to determine whether the increased levels of HMGB1 were related to the observed decreased relative abundance of *A muciniphila.* In Figure 1E, we found that there was a negative correlation between *A muciniphila* abundance and circulatory HMGB1 levels (Pearson's $r = -0.50$; R^2 COD = 0.255).

Exposure to GW chemicals is associated with blood-brain barrier tight junction protein dysregulation, and the changes persist five months after exposure

The study by Abou-Donia et al[14] suggests the presence of a leaky blood-brain barrier (BBB) among veterans who returned from the GW, and this may be a portal for immunostimulatory particles such as DAMPS and PAMPS to continuously fuel neuroinflammation. We studied the messenger RNA (mRNA) and protein expression levels of Claudin 5, the major tight junction protein in the complex that makes up the BBB. We found that GWP mice also exhibited significantly lower Claudin 5 mRNA and protein levels compared with vehicle control treated mice (CONT) $P = .042$ and $P = .03$; n = 6 (Figure 2A to C). Furthermore, we studied the levels of Claudin 5 in the BBB by observing colocalizations between Claudin 5 and CD31, a marker for endothelial cells that make up the lining of the blood vessels (Figure 2D and E). We found that there was a significant decrease in the number of colocalizations (yellow spots) constituting Claudin 5 and CD31 in GWP mice compared with controls (CONT) ($P = .04$; n = 6). This result points to the fact that at least one major component of BBB integrity is repressed at the protein level, paving the way for possible dysfunctional BBB, and this may lead to the passage of DAMPS such as HMGB1 leaking into the brain and triggering several immune responses.

GW chemical exposure is associated with persistent activation of microglia via the HMGB1-RAGE pathway resulting in increased reactive oxygen species and triggering of the NLRP3 inflammasome

Chronic neurological disorders such as AD and PD are characterized by activation of immune cells such as microglia, the

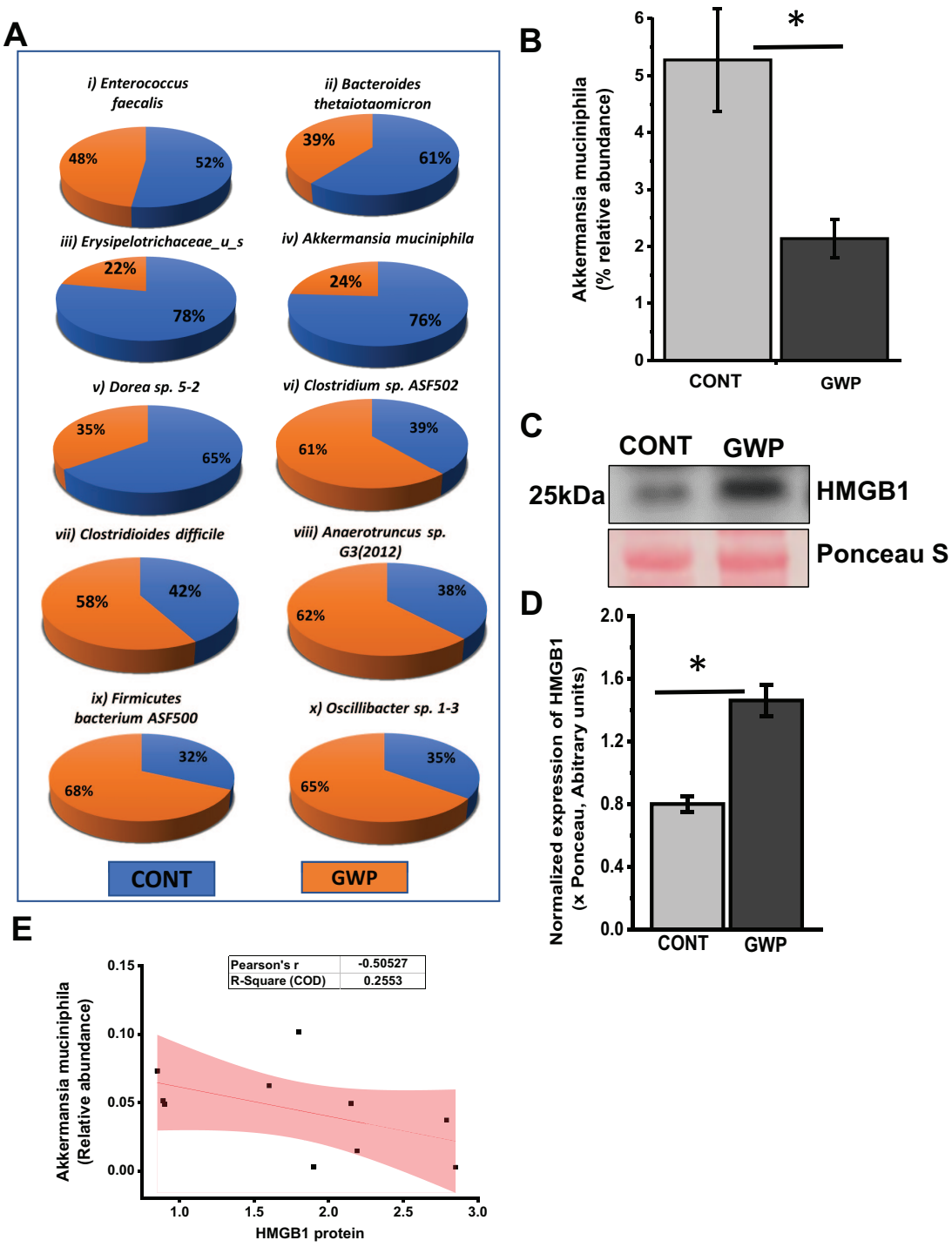

Figure 1. Exposure to GW chemicals results in decreased relative abundance of *Akkermansia muciniphila* and chronic high levels of circulatory HMGB1. (A) Percentage abundance of gut bacteria species. Percentage abundance of 10 most abundant species in the gut bacteriome are represented comparing GW chemical treated groups (GWP) to vehicle control treated group groups (CONT). Data are represented as the mean of 6 mice per group. (B) Percentage relative abundance of *A muciniphila.* Percentage relative abundance was determined from duplicate fecal samples of 5 mice per group treated with GW chemicals (GWP) compared with mice treated with vehicle control only (CONT). Data are represented as mean ± SEM (**P* < .05; n = 5). (C) Serum HMGB1 levels. Western blot of HMGB1 levels in serum for mice treated with GW chemicals (GWP) compared with mice treated with vehicle control (CONT) only. Data are represented as mean ± SEM. (D) Densitometry of HMGB1 immunoblots, normalized against Ponceau red (**P* < .05; n = 6). (E) Relationship between *A muciniphila* and circulatory HMGB1 levels. A correlative analysis was carried out to determine how *A muciniphila* is related to serum HMGB1 levels. We found a negative correlation between *A muciniphila* and serum HMGB1 levels (Pearson's $r = -0.50$; R^2 COD = 0.255 shaded area represents 95% confidence bands). GW indicates Gulf War; SEM, standard error of the mean; COD, coefficient of dipersion.

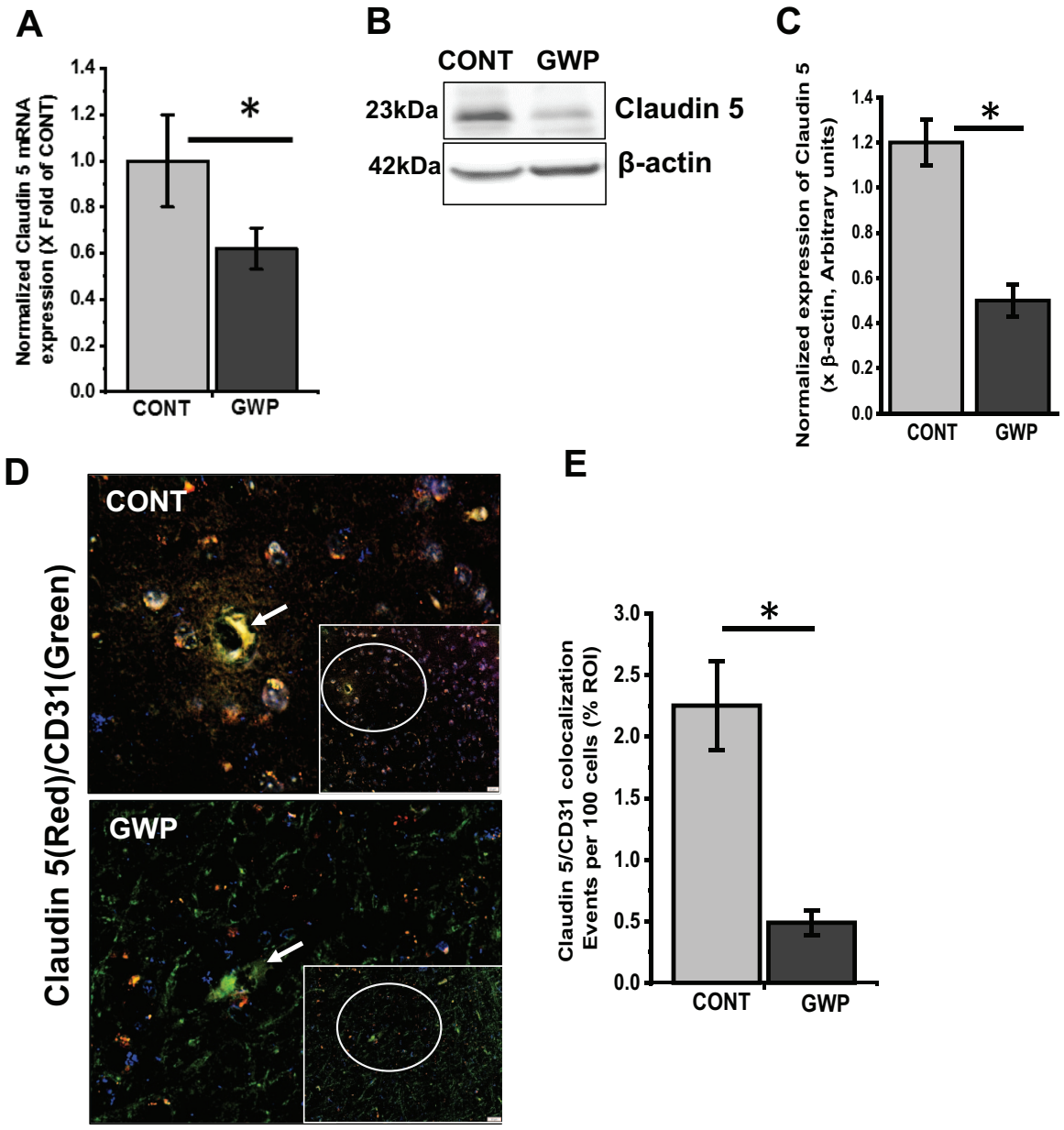

Figure 2. Exposure to GW chemicals is associated with altered Claudin 5 levels in the frontal cortex. (A) Claudin 5 mRNA levels in the frontal cortex: mRNA levels of Claudin 5 as studied by RTqPCR show that mice exposed to GW chemicals (GWP) had significantly decreased Claudin 5 mRNA levels compared with mice treated with vehicle control only (CONT). (B) Claudin 5 protein levels in frontal cortex. Western blot analysis of Claudin 5 protein levels in CONT and GWP treated mice. (C) Morphometry analysis of Claudin 5 immunoblots, normalized against β-actin (*$P < .05$; n = 6). Data are represented as mean ± SEM. (D) Representative immunofluorescence micrographs of the blood-brain barrier showing colocalization of tight junction protein Claudin 5 (labeled in red) and endothelial cell marker CD31 (labeled in green) as yellow spots around a BBB (magnification 60× and scale bar 10 µm) and DAPI stained nucleus (labeled in blue). See Supplemental Figure S2 for images of separate channels. Inset (magnification 40× and scale bar 20 µm) shows the whole micrograph field, from which the main image was obtained. (E) Quantitative morphometry analysis of colocalizations for every 100 cells represented as % ROI (*$P < .05$; n = 6). Data are represented as mean ± SEM. BBB indicates blood-brain barrier; GW, Gulf War; SEM, standard error of the mean; ROI, region of interest.

resident macrophages of the brain.[39] These cells may be activated by the presence of pathogens or DAMPS, such as HMGB1. We studied the protein expression levels of activated microglia marker TMEM119. The results showed that there was a significant increase in activated microglia in the frontal cortex of mice treated with GW chemicals (GWP) compared with controls (CONT) (*$P = .02$; n = 6) (Figure 3A and B) even after 3 months of exposure. Furthermore, we found that there was evidence of activated HMGB1-RAGE signaling, as indicated by colocalization events. Figure 3C and D shows a significantly high expression of RAGE protein levels (*$P = .04$; n = 6) and subsequent increased RAGE-HMGB1 colocalizations (*$P = .001$; n = 6) in GWP mice compared with controls (Figure 3E and F). RAGE is a receptor, which binds several ligands including HMGB1. Interaction of HMGB1-RAGE can lead to increasing reactive oxygen species (ROS)

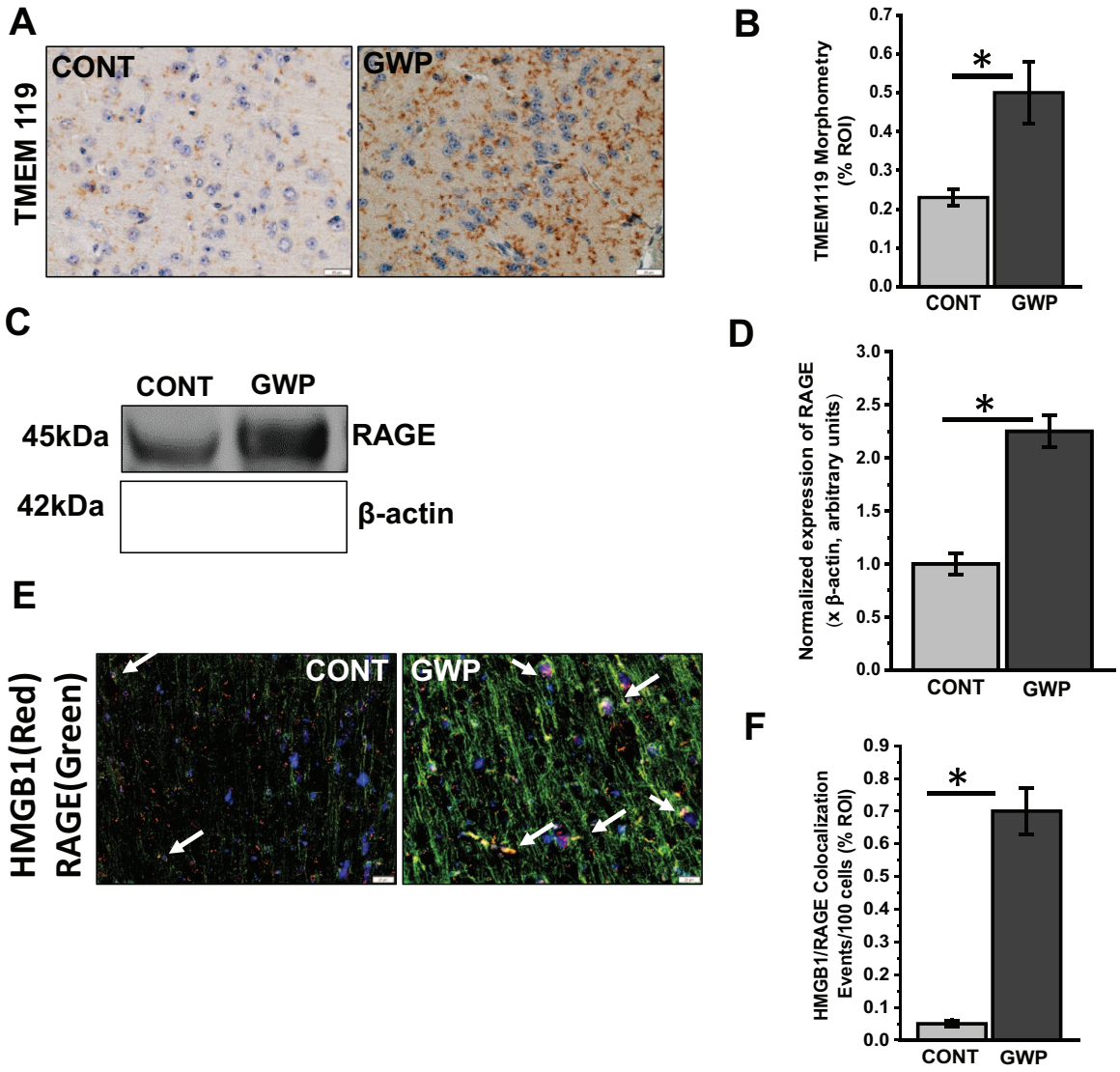

Figure 3. Activation of macrophages and associated HMGB1/RAGE complex formation. (A) TMEM119 immunoreactivity in frontal cortex. Representative immunohistochemistry micrographs of TMEM 119 reactivity in control (CONT) and GW chemical treated (GWP) mice (magnification 40× and scale bar 50 µm). (B) Morphometric analysis (represented as % ROI) obtained from 10 to 15 images from different microscopy fields from each mouse sample. Data are represented as mean ± SEM (*$P < .05$; n = 6). (C) Western blots of RAGE protein levels in the frontal cortex of GWP and CONT treated mice. (D) Morphometry analysis of all immunoblots normalized against β-actin (n = 5) (*$P < .05$; n = 6). Data are represented as mean ± SEM. (E) RAGE/HMGB1 complex formation. HMGB1 (labeled in red) and RAGE (labeled in green) and DAPI stained nucleus (labeled in blue) in GWP and CONT mice. Colocalizations are shown as yellow dots and marked with arrows in the micrographs (magnification 40× and scale bar 20 µm). See Supplemental Figure S3 for images of separate channels. (F) Morphometric analysis (represented as % ROI) obtained from 6 to 8 images from different microscopy fields from each mouse sample. Data are represented as mean ± SEM. (*$P < .05$; n = 6). GW indicates Gulf War; SEM, standard error of the mean; ROI, region of interest.

generation, eg, peroxynitrite, which reacts with tyrosine in proteins to form the stable adduct 3 nitrotyrosine. In Figure 4A and B, we detected significantly higher levels 3-nitrotyrosine (3NT) in GW chemical treated mice (GWP) compared with vehicle control treated mice (CONT); (*$P = .01$; n = 6). We also found that mice exposed to GW chemicals had significantly higher inflammasome activation compared with vehicle control treated mice (CONT) Figure 4C and D (*$P < .001$; n = 6). This activation was detected as yellow dots indicating a colocalization between the NLRP3 protein complex (labeled with red antibody) and the adapter protein ASC2 (marked with green), which facilitate the processing of pro-inflammatory cytokines from their basal inactive to more active form via protein cleavage.

Exposure to GW chemicals, decreased abundance of A muciniphila is associated with a persistently increased neuroinflammation and low levels of BDNF

Inflammasomes are large immune complexes found in several cell types and are responsible for processing the inflammatory cytokines IL-1β and IL-18 by cleaving them from their precursors.[40] Uncontrolled activation of these complexes can lead to chronic inflammation, as has been found in cancer, diabetes,

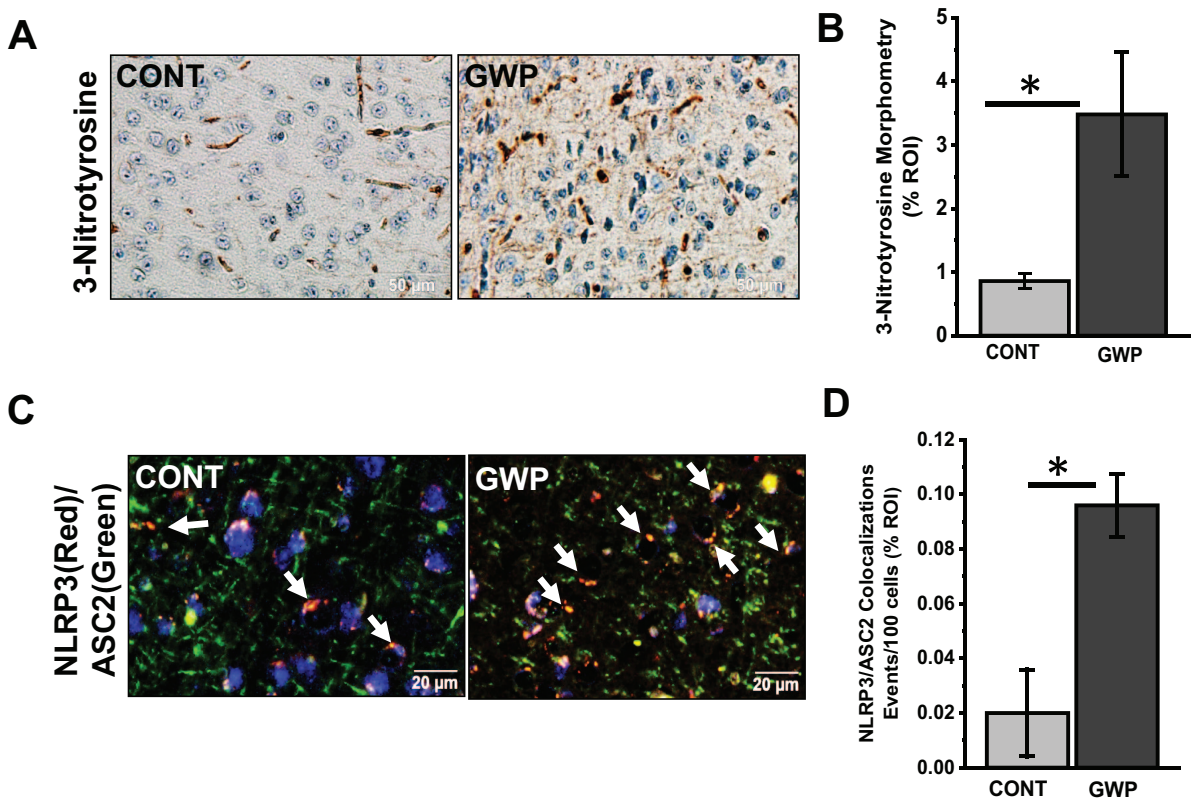

Figure 4. Increased ROS is associated with NLRP3 inflammasome activation. (A) 3-nitrotyrosine immunoreactivity in frontal cortex. Representative immunohistochemistry micrographs of 3-nitrotyrosine reactivity in control (CONT) and GW chemical treated (GWP) mice (magnification 40× and scale bar 50 µm). (B) Morphometric analysis (represented as % ROI) obtained from 10 to 15 images from different microscopy fields from each mouse sample. Data are represented as Mean ± SEM (*$P < .05$; n = 6). (C) Immunofluorescence micrographs showing activation of NLRP3 inflammasome protein. NLRP3 (labeled red) ASC2 (labeled in green) and DAPI stained nucleus (labeled in blue) in GWP and CONT mice. Colocalizations are shown as yellow dots and marked with arrows in the micrographs. See Supplemental Figure S4 for images of separate channels. Magnification 60× and scale bar 20 µm. (D) Morphometric analysis (represented as % ROI) obtained from 6 to 8 images from different microscopy fields from each mouse sample. Data are represented as mean ± SEM (*$P < .05$; n = 6). GW indicates Gulf War; ROS, reactive oxygen species; SEM, standard error of the mean; ROI, region of interest.

and neurodegenerative disease.[41-44] In our study, we found that NLRP3 inflammasome activation was also significantly associated with increased IL-1β (Figure 5A and B; $P = .001$, n = 6), IL-18 (Figure 5C and D; $P = .021$, n = 6), and IL-6 (Figure 5E and F; $P = .01$, n = 6) protein levels in GWP mouse frontal cortex when compared with mice treated with only vehicle control (CONT) (Figure 5A and F). Neurodegenerative diseases are often characterized by neuroinflammation, accompanied by decreased levels of neurotrophins.[18,45] Similarly, in this study, we observed that mice which were treated with GW chemicals had significantly lower levels of the neurotrophin BDNF when compared with mice treated with only vehicle control (CONT) (Figure 5G and H, $P = .018$ and Figure 5I and J, $P = .001$ n = 6).

A muciniphila relative abundance correlates with BDNF levels and persistent neuroinflammation

A high abundance of *A muciniphila* has been linked to decreased inflammation in chronic diseases.[38,46,47] To study whether the host bacteria's abundance played a role in affecting chronic inflammation and sustained BDNF levels in our model of GWI, we carried out correlative analyses to determine whether there were statistically significant relationships between the bacterial abundance, inflammation, and BDNF levels. The results showed that there was a significant positive correlation between BDNF levels and abundance of *A muciniphila* (Pearson's $r = 0.83$, R^2 COD = 0.73; $P = .0024$), and a significant negative correlation with IL-1β protein levels (Pearson's $r = -0.684$; R^2 COD = 0.46; $P = .02$) (Figure 6A and B). Shaded area represents 95% confidence bands.

Deletion of NLRP3 is protective against persistent systemic and neuroinflammation and is associated with an increase in BDNF levels

To study the role of NLRP3 in driving inflammation and lowering BDNF levels, we treated mice lacking NLRP3 with GW chemicals and then subjected them to our experimental conditions for 20 weeks or 5 months. We then studied the protein levels of IL-1β, IL-18, IL-6, and BDNF by western blot analysis and immunohistochemistry. Our results show that there were significantly lower levels of IL-1β (Figure 7A and E; $P = .006$, n = 6, IL-18 (Figure 7B and F; $P = .01$, n = 6), IL-6 (Figure 7C and G; $P = .015$, n = 6), and increased BDNF

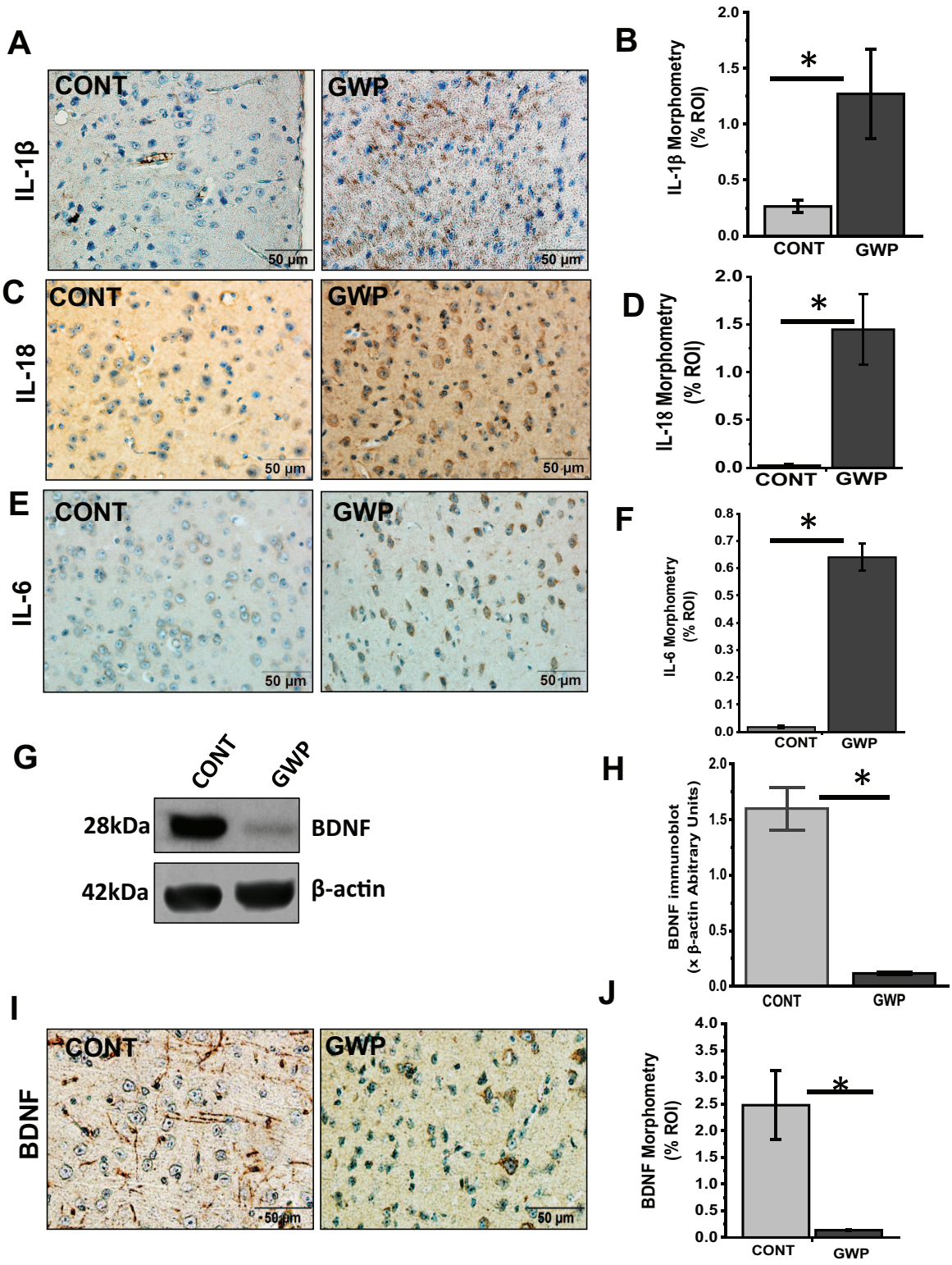

Figure 5. GW chemical exposure is associated with chronic neuroinflammation and decreased brain-derived neurotrophic factor (BDNF) levels in frontal cortex.

(A, C, E) Immunohistochemistry micrographs of frontal cortex tissues of GWP and CONT treated mice showing immunoreactivity of IL-1β, IL-18, and IL-6 (magnification 20× and scale bar 50 μm). (B, D, F) Morphometric analysis (as % ROI) obtained from 10 to 15 images from different microscopic fields from each mouse sample. (*$P < .05$; n = 6). Data are represented as mean ± SEM. (G) Western blots of BDNF protein levels in the frontal cortex of GWP and CONT treated mice. (H) Morphometry analysis of immunoblots normalized against β-actin (n = 5) (*$P < .05$; n = 5). Data are represented as mean ± SEM. (I) BDNF immunoreactivity. Representative immunohistochemistry micrographs showing BDNF in frontal cortex tissues of GWP and CONT treated mice (magnification 40× and scale bar 50 μm). (J) Morphometric analysis (represented as % ROI) obtained from 10 to 15 images from different microscopy fields from each mouse sample (*$P < .05$; n = 6). Data are represented as mean ± SEM. BDNF indicates brain-derived neurotrophic factor; GW, Gulf War; SEM, standard error of the mean; ROI, region of interest.

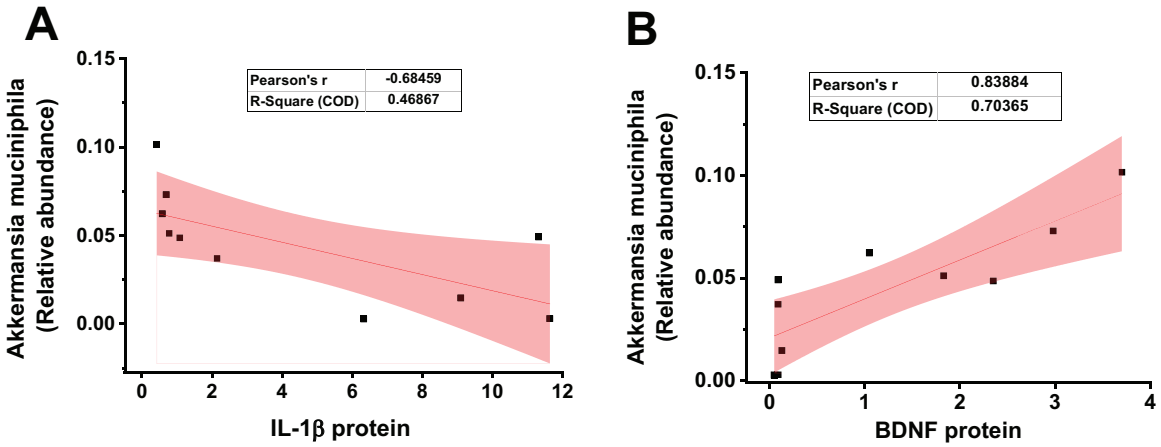

Figure 6. The decreased relative abundance of *A muciniphila* correlates with IL-1β and BDNF levels in the frontal cortex. (A) Correlation between *A muciniphila* and IL-1β levels in GW chemical (GWP) and vehicle control (CONT) treated mice. We carried out a linear regression analysis to determine the relationship between IL-1β and *A muciniphila* in GWP and CONT mice. There was a negative correlation between *A muciniphila* and IL-1β in the FC (Pearson's $r = -0.68$; R^2 COD = 0.46 and $P = .02$). (B) Correlation between *A muciniphila* and BDNF levels in GW chemical (GWP) and vehicle control (CONT) treated mice. We carried out a linear regression analysis to determine the relationship between BDNF and *A muciniphila* in GWP and CONT mice. There was a positive correlation between *A muciniphila* relative abundance and BDNF levels (Pearson's $r = 0.83$, R^2 COD = 0.7 and $P = .0024$). BDNF indicates brain-derived neurotrophic factor; GW, Gulf War; COD, coefficient of dispersion.

(Figure 7D and H; $P = .007$, and Figure 7I and J; $P = .017$, n = 6) levels in the frontal cortex of NLRP3 KO mice treated with GW chemicals (GWP-NLRP3KO), when compared with wild type mice treated with GW chemicals (GWP). Furthermore, we studied systemic inflammation levels in the three groups of mice by analyzing serum IL-1β (Figure 8A; $P = .046$, n = 6), TNF-α (Figure 8B; $P = .032$, n = 6) and IL-6 (Figure 8C; $P = .042$, n = 6) levels using an ELISA. Our results show that there is significantly lower inflammation in NLRP3KO mice treated with GW chemicals (GWP-NLRP3KO) compared with mice treated with GW chemicals (GWP).

Discussion

In our previous studies, we reported that there was a general alteration in microbiome accompanied by endotoxemia, a leaky gut, and inflammation in different organs such as the small intestine, brain, and liver.[30-32] We found significant increases in phyla Firmicutes and Tenericutes over Bacteriodetes in GW chemical exposed mice when compared with controls.[31] However, it was not clear whether these alterations persisted long after GW chemical exposure or would eventually resolve over time through the repopulation and reconstitution of the host microbiome. We also were eager to study the mechanisms that would connect the altered microbiome and the persistent inflammatory changes in the intestine and the neural-immune network. In this study, mice were exposed to the GW chemicals and allowed to ad libitum diet and water for 20 weeks. This was to simulate the period of exposure (during the GW) and the subsequent period following their return from the war. We found that exposure to GW chemicals (the pesticide permethrin and prophylactic drug PB) in mice resulted in persistent pathology characterized by the low abundance of *A muciniphila*, high circulatory HMGB1 levels, BBB dysfunction, neuroinflammation, and lowered neurotrophin BDNF levels. Our findings and the proposed mechanism are summarized as a schematic illustration in Figure 9.

We report that exposure to GW chemicals caused a decrease in *A muciniphila* or resulted in conditions that favor other bacteria populations repopulate over *A muciniphila* (Figure 1A). *A muciniphila* is a mucin degrading bacterium which exists as part of the normal human gut flora and is abundant in healthy individuals.[47-50] In recent years, the herein reported bacterium is emerging as an important probiotic which can be consumed to improve health.[51] This bacterium was found to improve ulcerative colitis in mice[52] and restored colonic mucus layer thickness with decreased inflammation in aging mice.[53] In another study, the abundance of this bacterium inversely correlated with inflammation and altered lipid metabolism in obese mice.[51] Although the mechanism by which *A muciniphila* promotes these health benefits is not fully understood, studies report that the bacterium strengthens gut barrier integrity through its association with enterocytes and also produces high amounts of anti-inflammatory cytokine IL-8.[48,54] It is possible that the low levels of this bacterium in the gut compromised gut barrier integrity, a condition that we have observed in our previous acute models of GWI.[29,31] This condition of compromised gut barrier integrity has also been reported among veterans who suffer from gastrointestinal problems in GWI.[55] Moreover, a recent study by Janulewicz et al[56] showed that GWI afflicted veterans with gastrointestinal disturbances present gut dysbiosis among bacteria of the phylum Verrucomicrobia. Interestingly, *A muciniphila* belongs to the same phylum. Another study found that *A muciniphila* treatment normalized diet-induced metabolic endotoxemia, adiposity, and the adipose tissue marker CD11c in obese mice, which otherwise had increased inflammatory indicators in the intestine and aided in

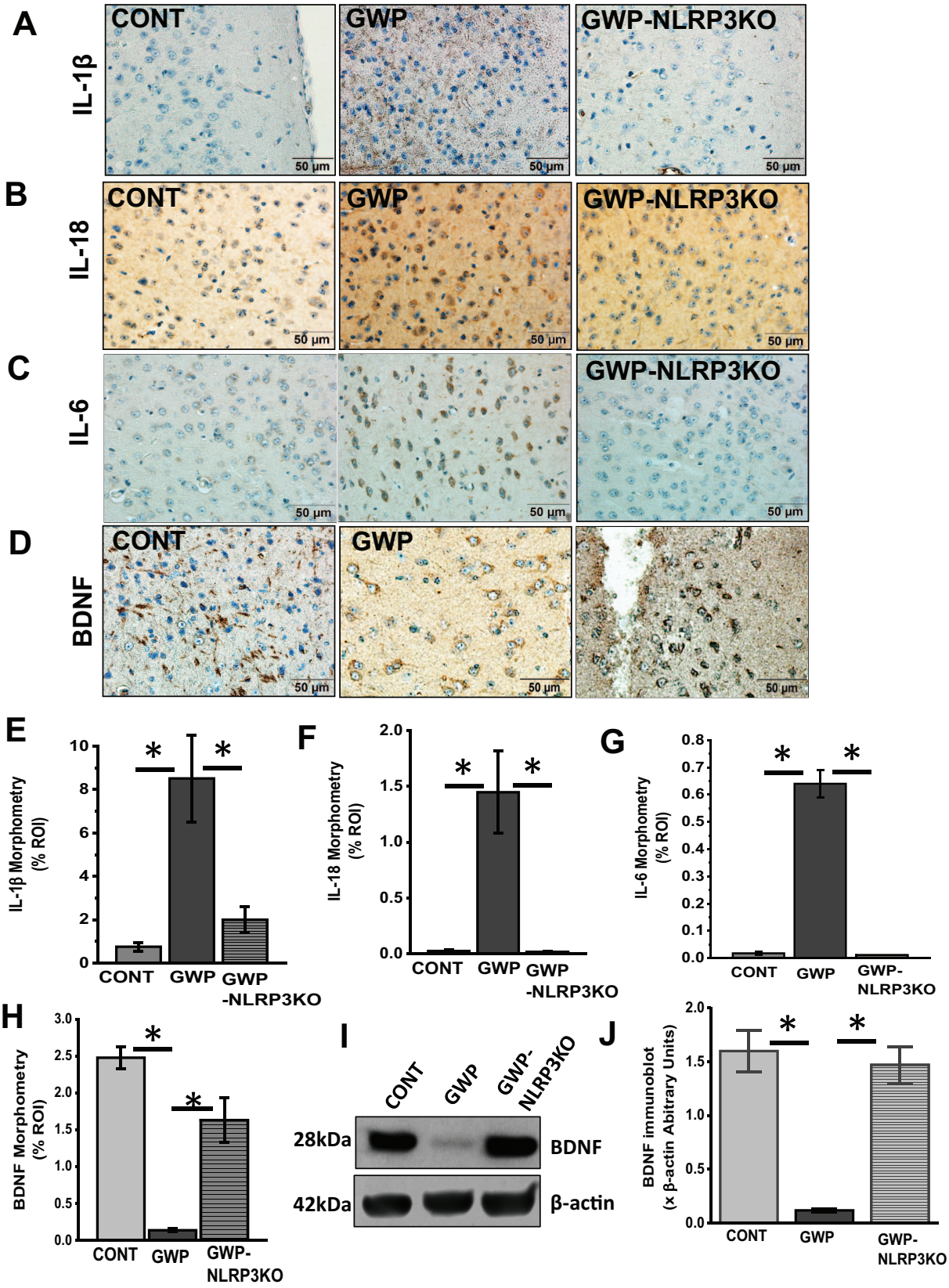

Figure 7. Deletion of NLRP3 is associated with decreased neuroinflammation and lower BDNF levels. (A to D) IL-1β, IL-18, IL-6, and BDNF immunoreactivity in the frontal cortex. Representative immunohistochemistry micrographs showing IL-1β, IL-18, IL-6, and BDNF, respectively, in frontal cortex tissues of GW chemical treated (GWP), GW chemical treated NLRP3KO (GWP-NLR3KO), and vehicle control (CONT) treated mice (magnification 40× and scale bar 50 μm). (E to H) Morphometric analysis (represented as % ROI) obtained from 10 to 15 images from different microscopy fields from each mouse sample. Data are represented as mean ± SEM. (*$P < .05$, n = 6). (I) Western blots of BDNF protein levels in the frontal cortex of GWP and CONT treated mice. (J) Morphometry analysis of all immunoblots normalized against β-actin. Data are represented as mean ± SEM (*$P = .05$, n = 5). BDNF indicates brain-derived neurotrophic factor; GW, Gulf War; SEM, standard error of the mean.

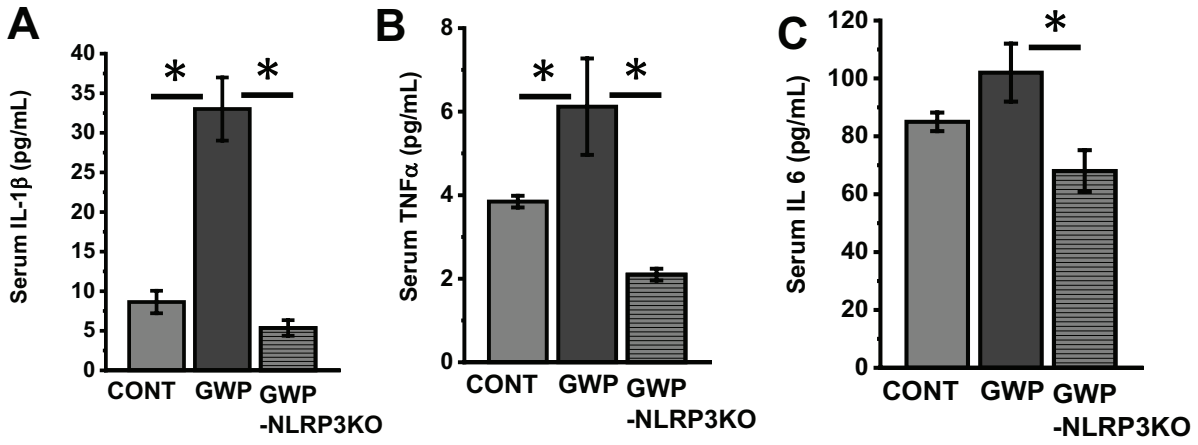

Figure 8. Deletion of NLRP3 is associated with decreased serum cytokine levels. (A to C) Serum cytokine levels. IL-1β, TNF-α, and IL-6 levels, respectively, determined by ELISA in the serum of vehicle control (CONT), GW chemical treated (GWP) and GW chemical treated NLRP3KO mice (GWP-NLRP3KO). Data are represented as mean ± SEM (*$P < .05$, n = 6). GW indicates Gulf War; SEM, standard error of the mean.

the metabolic disease development.[46] Similarly, our studies of GWI mouse models have consistently found that altered levels of tight junction proteins in the gut was associated with increase in endotoxins and DAMPs such as HMGB1 and inflammation that was related to an alteration of gut microbiome abundance.[29,32] Notably, mice in the present study (using a persistence model of GWI) that were exposed to similar chemicals showed a slight increase in serum endotoxin levels (low-level endotoxemia consistent with an obesity phenotype) in GW chemical treated mice when compared with vehicle control treated mice (Supplemental Figure S1). In addition, a significant increase in circulatory HMGB1 levels was observed in GW chemical exposed mice which negatively correlated with *A muciniphila* relative abundance (Figure 1C to E) suggesting that a sustained and consistent low inflammatory trigger was closely associated with a persistent change in the microbiome and decreased *A Muciniphila* abundance in these mice. Furthermore, the persistence of systemic inflammatory indicators such as pro-inflammatory cytokines, eg, IL-1β, IL-6, and TNF-α (Figure 8A to C), endotoxin levels and serum HMGB1 for such a long period that failed to ease even 20 weeks after exposure (equivalent to > 20 human years) and its connection with an altered microbiome triggered our interest to study their effects on neuronal structures and their networks.

Before we could study the neuroinflammatory indicators for persistence, we needed to assess the integrity of the BBB, a vital interface of neuronal physiology and pathology. Interestingly, our results showed that the expression of Claudin 5, a critical tight junction protein of the BBB in the brain, was decreased in the frontal cortex of mice treated with GW chemicals compared with controls (Figure 2A to E). This protein, together with others such as Claudin 1, zona occludens, and occludins make up tight junctions in the BBB. The BBB is a selective barrier found at the interface of blood vessels in the brain and brain tissue. It is made of a single cell layer of endothelial cells, astrocyte, and pericytes. Its unique properties allow it to tightly regulate the movement of particles between the circulation and brain tissue.[57,58] Claudin 5 and other tight junction proteins are found between adjacent endothelial cells of blood vessels and help to anchor these cells to create a tightly regulated selective barrier that allows the passage of particles between the blood and the brain.[59] Low levels of this protein have been found in neurodegenerative and neuroinflammatory diseases such as AD, PD, and schizophrenia.[60,61] We found decreased mRNA and protein levels of this protein in GWP mice compared with controls (Figure 2A to E). This provides strong evidence that at least one key component of the BBB is dysregulated and this possibly compromised the barrier's integrity, likely caused by the serum mediators endotoxins, HMGB1, and pro-inflammatory cytokines causing it to become leaky. However, we have no direct evidence of such an event in an in vitro experimental setup using BBB endothelial cells. We hypothesized that this leaky BBB allowed the passage of unwanted particles such as DAMPs and PAMPS, HMGB1, as one such example, which we found to be greatly increased in the serum. It is also worth noting that even though serum endotoxin levels are not significantly higher in GWP mice compared with controls, even low levels of endotoxins over a long time can be a toxic stimulus to the body and may contribute to observed pathology.[62,63]

HMGB1 is a DAMP known to trigger proinflammatory pathways through toll-like receptors (TLRs), eg, TLR4, and through the receptor for advanced glycation end products (RAGE).[64,65] We found that there was increased activation of microglia in GWP mice compared with controls (Figure 3A and B) with an increased expression of RAGE receptors in the frontal cortex (Figure 3C and D). We also detected HMGB1/RAGE complex formation using immunofluorescence microscopy, which may indicate activation of RAGE signaling (Figure 3E and F). The activation of this pathway is known to result in the transcription of pro-inflammatory cytokines and the generation of ROS[66] such as nitric oxide. High levels of nitric oxide in the presence of superoxide could result in the formation of

peroxynitrite, a potent ROS which attacks tyrosine to form 3 nitrotyrosine denaturing them and rendering them dysfunctional. We found significantly higher levels of ROS in our GWP mice compared with controls (Figure 4A and B). High amounts of ROS are a known trigger of the NLRP3 inflammasome.[67] Inflammasomes are large protein complexes that are assembled in response to infections, etc, and are involved in the processing of pro-inflammatory cytokines such as IL-1β and IL-18. We found that mice treated with GW chemicals had higher expression of NLRP3/ASC2 complex formation compared with controls (Figure 4C and D). Although inflammasomes are triggered as a defense mechanism, chronic activation of these complexes has been implicated in conditions characterized by chronic low-grade inflammation such as diabetes, PD, and ALS.[68,69] This activation of the NLRP3 inflammasomes was followed by increased IL-1β and IL-18 levels in the brain (Figure 5A to D), which might contribute to the persistent neuroinflammation in GWI.

In neurological diseases, inflammation has been associated with poor neuronal health, with fewer neurons, decreased neuronal plasticity, and growth.[70] This in part is due to low levels of neurotrophins such as BDNF. BDNF is a neurotrophin produced by neurons and is involved in neuronal growth, survival, and plasticity.[23] Studies by Guan and Fang, and another by Lapchak, suggest that increased IL-1β levels interfered with BDNF synthesis,[71,72] while Tong et al showed that increased IL-1β interfered with BDNF signaling through the PI3K/AKT pathway by preventing its activation of AKT. The above mechanisms resulted in decreased growth and survival of neurons.[73] In this study, we report low levels of BDNF in the frontal cortex of mice, which were treated with GW chemicals compared with controls even 5 months post-exposure (Figure 5F and G). We also studied any correlations that BDNF, IL-1β with *A muciniphila* relative abundance may have to connect the intestinal, microbiome changes, and neuronal levels of these mediators referenced above. In Figure 6A and B, we show that *A muciniphila* relative abundance correlated negatively with IL-1β levels and positively with BDNF levels. The above result indicates that *A muciniphila* relative abundance might have played a role in modulating neuroinflammation and neurotrophin levels in GWI as has been the case in obesity and other diseases described previously.[46] This could be through the bacterium's production of anti-inflammatory compounds that counter inflammation or modulation of the intestinal barrier integrity. However, more studies need to be done to determine the exact mechanism. Finally, to study the role of NLRP3 inflammasome in contributing to neuroinflammation that persists for a prolonged period and its association with increased abundance of Akkermansia, we used a mouse model with the systemic knockout of NLRP3 gene (KO mouse). The results found that the deletion of this gene was associated with increased BDNF levels and protected the mice from neuroinflammation (Figure 7A to J). Deleting NLRP3 is protective

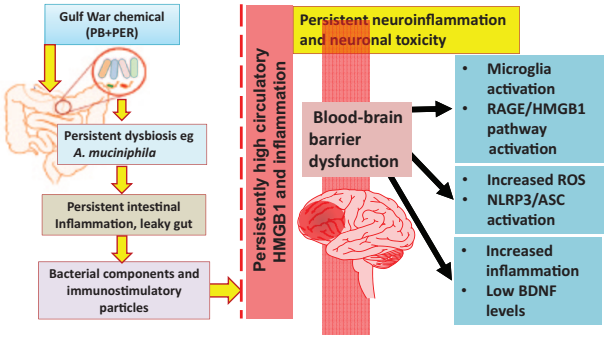

Figure 9. Schematic illustration of the proposed mechanism for persistent neuroinflammatory pathology in current study.

through preventing inflammasome activation and subsequent processing of pro-inflammatory cytokines and distinctly proves that the persistent inflammation in GWI chemical exposed mice that had altered *A muciniphila* abundance is due to NLRP3-mediated inflammasome activation though there may be multiple molecular mediators for triggering such an activation. Our current study identifies some of these mediators such as gut-derived endotoxins, HMGB1, or peroxynitrite, to name a few. Still, the molecular mechanism of such a trigger remains speculative currently.

In conclusion, we report that persistence of GWI inflammatory symptoms is characterized by low relative abundance of *A muciniphila* and chronic high circulatory HMGB1 levels which trigger NLRP3 mediated neuroinflammation and decreased levels of neurotrophins through RAGE signaling as shown in Figure 9. These findings not only provide insight into the mechanism of persistent neurological disturbances in GWI but also provide possible therapeutic targets. First, the microbiome can be targeted through replacement or enhancing of *A muciniphila* gut bacterial populations and other significantly lowered probiotic bacteria, and second, through therapies that target RAGE signaling or NLRP3 inflammasomes to relieve persistent inflammation and improve quality of life of veterans who suffer from GWI veterans.

Acknowledgements

We are grateful to the Instrument Resource Facility (University of South Carolina School of Medicine) and AML Labs (Baltimore MD) for providing excellent technical services. We are also thankful to Cosmos ID (Microbial Genomic Platform, Rockville, MD, USA) for their analysis of the bacteriome.

Author Contributions

SC conceived research, SC ,DK and DB designed research. DK, DB, RS, AM, PS performed research. KS, PJ, SL, RH, NK and SC analyzed and interpreted data. DK, DB and SC drafted

manuscript, SC wrote, edited and approved final version of the manuscript.

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Supplemental Material

Supplemental material for this article is available online.

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HHS Public Access

Author manuscript

Neuropharmacology. Author manuscript; available in PMC 2020 August 03.

Published in final edited form as:

Neuropharmacology. 2020 July ; 171: 108073. doi:10.1016/j.neuropharm.2020.108073.

Acetylcholinesterase inhibitor exposures as an initiating factor in the development of Gulf War Illness, a chronic neuroimmune disorder in deployed veterans

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Abstract

Gulf War Illness (GWI) is a chronic multi-symptom disorder, characterized by symptoms such as fatigue, pain, cognitive and memory impairment, respiratory, skin and gastrointestinal problems, that is experienced by approximately one-third of 1991 Gulf War veterans. Over the nearly three decades since the end of the war, investigators have worked to elucidate the initiating factors and underlying causes of GWI. A significant portion of this research has indicated a strong correlation between GWI and exposure to a number of different acetycholinesterase inhibitors (AChEIs) in theater, such as sarin and cyclosarin nerve agents, chlorpyrifos and dichlorvos pesticides, and the anti-nerve agent prophylactic pyridostigmine bromide. Through studying these exposures and their relationship to the symptoms presented by ill veterans, it has become increasingly apparent that GWI is the likely result of an underlying neuroimmune disorder. While evidence indicates that AChEIs are a key exposure in the development of GWI, particularly organophosphate AChEIs, the mechanism(s) by which these chemicals instigate illness appears to be related to "off-target", non-cholinergic effects. In this review, we will discuss the role of AChEI exposure in the development and persistence of GWI; in particular, how these chemicals, combined with other exposures, have led to a chronic neuroimmune disorder.

This article is part of the special issue entitled 'Acetylcholinesterase Inhibitors: From Bench to Bedside to Battlefield'.

Keywords

Gulf war illness; Acetylcholinesterase inhibitor; Neuroimmune; Neuroinflammation

1. Introduction

Acetylcholinesterase inhibitors (AChEIs) comprise a class of chemicals that can be generally categorized as therapeutics, pesticides, or nerve agents. During the 1991 Gulf War,

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soldiers had the potential to be exposed to all three of these categories of AChEIs: the pesticides chlorpyrifos (CPF) and dichlorvos (DDVP) were regularly and pervasively used for pest control, pyridostigmine bromide (PB) was prescribed to be taken every 8 h as a prophylactic against potential nerve agent exposure, and the demolition of ammunition storage facilities released sarin and cyclosarin nerve agents and potentially exposed veterans to these chemical weapons (Tuovinen et al., 1999; Institute of Medicine (US) Committee on Health Effects Associated with Exposures During the Gulf War, 2000; Research Advisory Committee on Gulf War Veteran' Illnesses, 2008; White et al., 2016). Exposure to AChEIs has been repeatedly implicated as the potential cause of Gulf War Illness (GWI), a chronic multi-symptom disorder affecting nearly one-third of the veterans that returned from the 1991 conflict, by both epidemiological (Sullivan et al., 2003; Research Advisory Committee on Gulf War Veteran' Illnesses, 2008; Steele et al., 2012; Kerr, 2015; White et al., 2016; Sullivan et al., 2018) and preclinical studies (Henderson et al., 2001; Amourette et al., 2009; Lamproglou et al., 2009; Abdullah et al., 2012; Abdullah et al., 2013; Parihar et al., 2013; Hattiangady et al., 2014; Ojo et al., 2014; Nutter et al., 2015; O'Callaghan et al., 2015; Zakirova et al., 2015; Abdullah et al., 2016; Cooper et al., 2016; Phillips and Deshpande, 2016; Pierce et al., 2016; Alhasson et al., 2017; Emmerich et al., 2017; Flunker et al., 2017; Locker et al., 2017; Shetty et al., 2017; Zakirova et al., 2017; Ashbrook et al., 2018; Carreras et al., 2018; Cooper et al., 2018; Koo et al., 2018; Macht et al., 2018; Miller et al., 2018; Phillips and Deshpande, 2018; Seth et al., 2018; Macht et al., 2019; Michalovicz et al., 2019). Acute exposure to toxic levels of AChEIs, particularly the organophosphate (OP) compounds like the pesticides and nerve agents, has a number of systemic effects (i.e. salivation, lacrimation, urination, defecation, gastrointestinal upset, emesis, miosis [SLUDGEM]) and can cause seizures. However, it is the long-term neurological symptoms associated with exposure to these compounds that harmonize with many of the long-lasting symptoms of GWI: fatigue, pain, mood disorders, cognitive and memory impairment (Fukuda et al., 1998; Steele, 2000; Haley et al., 2001; Golomb, 2008; Sullivan et al., 2003; Maule et al., 2018). Furthermore, the similarity of these symptoms to those of sickness behavior, the adaptive behavioral response elicited by illnessor infection-induced neuroinflammation (Dantzer and Kelley, 2007; Dantzer et al., 2008), has suggested that GWI is the result of an underlying chronic neuroimmune disorder; a hypothesis that has been supported by both preclinical animal (O'Callaghan et al., 2015; Zakirova et al., 2016; Alhasson et al., 2017; Locker et al., 2017; Shetty et al., 2017; Ashbrook et al., 2018; Carreras et al., 2018; Kodali et al., 2018; Koo et al., 2018; Macht et al., 2018, 2019; Miller et al., 2018; Hernandez et al., 2019; Joshi et al., 2019; Madhu et al., 2019; Michalovicz et al., 2019) and clinical studies (Broderick et al., 2013; Parkitny et al., 2015; White et al., 2016; Coughlin, 2017; Abou-Donia et al., 2017; Georgopoulous et al., 2017; Alshelh et al., 2020). In spite of the fact that the ACh signaling facilitated by AChE inhibition is typically anti-inflammatory (Pavlov et al., 2003; Pavlov and Tracey, 2005), several preclinical studies have directly shown a connection between GWI-relevant AChEI exposures and neuroinflammation (Ojo et al., 2014; O'Callaghan et al., 2015; Locker et al., 2017; Ashbrook et al., 2018; Koo et al., 2018; Miller et al., 2018). Moreover, while there are many hurdles to directly evaluate neuroinflammation in living humans, a recent clinical Positron Emission Tomography (PET) study found strong signals for the neuroinflammatory marker, 18 kDa transducer protein (TSPO), throughout the cortex of veterans with GWI compared to

controls, providing the first direct, *in vivo* data to show neuroinflammation in the illness (Alshelh et al., 2020).

In this review, we will discuss the three major groups of AChEIs that soldiers were exposed to during the 1991 Gulf War that have been associated with GWI: nerve agents, pesticides, and pyridostigmine bromide. In particular, we will focus on discussing the preclinical body of data that has supported the hypothesis that GWI is the result of an underlying neuroinflammatory/neuroimmune disorder. Lastly, we will discuss emerging evidence that suggests that this neuroimmune dysfunction is the result of persistent, non-cholinergic effects of AChEI exposure.

1.1. Acetylcholinesterase inhibitor exposures in GWI

The emergence of a chronic multi-symptom disorder in veterans immediately following the 1991 Gulf War raised questions regarding the possibility that harmful exposures occurred during the war (Pennisi, 1996). Soldiers that were deployed during the 1991 Gulf War were exposed to a number of conditions that could have increased their risk for negative health impacts, including pesticides, nerve agent, oil well fire smoke, depleted uranium munitions, prophylactic drugs and vaccinations, paints, and psychological and/or physiological stress (White et al., 2016). However, the prevalence of neurological and neuropsychological symptoms among veterans with GWI has fueled an interest in the neurotoxicant exposures that may have been experienced in theater (White et al., 2016). Among these neurotoxicants, there was the potential for and, in some cases, documentation of, the exposure of soldiers to both irreversible and reversible AChEIs during deployment. The delineation between these two subcategories of AChEIs is important because the perceived risks associated with irreversible OP AChEIs, i.e. those pesticides and nerve agents that permanently bind and inactivate the enzyme, is greater than the reversible AChEIs (including pharmaceuticals) that only modulate enzyme activity through on-off binding dynamics. In this section, we will discuss both subclasses of AChEIs that have been investigated in GWI and how exposure to these compounds may contribute to the associated chronic neuroinflammatory dysfunction.

1.2. Sarin, cyclosarin, and nerve agent surrogates

Exposure to sarin and/or cyclosarin nerve agent, both OP AChEIs, was a very real risk to soldiers deployed during the 1991 Gulf War. Interviews with veterans of the 1991 Gulf War have indicated that many of them recall hearing chemical alarms in camp during deployment (Haley and Kurt, 1997; Kurt, 1998; Haley and Tuite, 2013; Chao et al., 2016). Additionally, several investigations have determined that the destruction of multiple munitions storage sites by U.S. troops released a plume containing both sarin and cyclosarin that is predicted to have exposed upwards of 100,000 soldiers (Institute of Medicine (US) Committee on Health Effects Associated with Exposures During the Gulf War, 2000; Research Advisory Committee on Gulf War Veteran' Illnesses, 2008). Interestingly, a survey of soldiers that were in proximity to one of these detonation sites, Khamisiyah, found that the majority of respondents did not report any acute cholinergic symptoms (Institute of Medicine (US) Committee on Health Effects Associated with Exposures During the Gulf War, 2000). However, many studies of sick veterans have uncovered an association between potential nerve agent exposures and GWI presentation (see review in Golomb, 2008; White et al.,

2016). While nerve agent exposure has been associated with incidence of high blood pressure, diabetes, arthritis and chronic bronchitis in GWI (Zundel et al., 2019), the observations of MRI-assessed brain structural abnormalities like decreased gray and white matter volumes and increased ventricle size (Heaton et al., 2007; Chao et al., 2010, 2011, 2014, 2015; Chao and Zhang, 2018), as well as poor neurobehavioral performance (White et al., 2001; Proctor et al., 2006; Toomey et al., 2009) and an increased risk for brain cancer (Bullman et al., 2005) are more indicative of the long-term brain effects of nerve agent exposure. These studies combined with the result of the Department of Defense survey suggest that GWI may be the result of low-level exposures to sarin and/or cyclosarin that went undetected due to a lack of immediate/acute neurotoxic effects.

Though posing an obvious neurotoxicological risk, only a handful of preclinical studies have investigated the role of potential sarin exposure in the development of GWI. A few of these studies have investigated the potential outcomes of low-dose, sub-lethal exposure(s) to sarin or sarin surrogates alone in relationship to GWI symptomology. While subclinical inhalation of sarin has been demonstrated to be capable of producing signs of immunosuppression, such as reduced T-cell responses and reduced corticosterone levels (Henderson et al., 2001; Kalra et al., 2002), as well as autonomic imbalance and cardiomyopathy (Shewale et al., 2012), these studies do not directly correlate with the neuroimmune dysfunction hypothesis for GWI. However, repeated, low-dose exposure to the sarin surrogate diisopropyl fluorophosphate (DFP) has been shown to instigate depression, cognitive impairment, and neuronal damage (Phillips and Deshpande, 2016, 2018). In these studies, rats were injected with 0.4–0.5 mg/kg, s.c. DFP once a day for 5 days and, when assessed three to six months after exposure, demonstrated memory impairments in novel object recognition and object location tasks, signs of anxiety and depression as indicated by increased immobility time in the forced swim test, reduced sucrose preference, and reduced time in the open arms of the elevated plus maze. These behavioral observations were concurrent with increased levels of neuronal calcium and neuronal cell damage in the hippocampus. In addition, 14-day exposure to the same dose of DFP was found to result in impaired axonal transport (Naughton et al., 2018). While there were no direct evaluations of neuroinflammation or neuroimmune dysfunction in these studies, an expanding body of research has suggested an association between neuroinflammation and depression (Walker et al., 2013), as well as axonal transport deficits (Errea et al., 2015). However, these results not only support a role for nerve agent exposure in the development of GWI symptoms, but also demonstrate that these low-dose exposures are capable of producing long-lasting effects that can translate to the decades of persistent illness experienced by veterans with GWI.

Deployed soldiers were exposed to significant physiological stressors such as exercise and extreme temperatures (Young et al., 1992; Sapolsky, 1998; Sullivan et al., 2003) that had the potential to interact with or modulate the responses to chemical exposures. As such, several studies investigating the contribution of nerve agent exposure to GWI have found positive correlations to illness symptomology when sarin surrogates have been combined with a stressor (O'Callaghan et al., 2015; Locker et al., 2017; Ashbrook et al., 2018; Koo et al., 2018; Miller et al., 2018; Craddock et al., 2018; Michalovicz et al., 2019; Belgrad et al., 2019). In this GWI rodent model, a single dose of DFP is preceded by a chronic (4–7 day) exposure to exogenous corticosterone (CORT) provided in the drinking water (200 mg/L) to

mimic the high physiological stress experienced by soldiers during deployment. Though currently acute in scope, the extensive evaluation of this model has strongly supported the role of neuroimmune dysfunction as the underlying cause of GWI showing that this combination of exposures results in CORT priming of the DFP neuroinflammatory response leading to significantly increased inflammatory cytokine mRNA throughout the brain, including cortex, hippocampus, and striatum (O'Callaghan et al., 2015; Locker et al., 2017; Koo et al., 2018; Miller et al., 2018), along with alterations in neuroimmune signaling via histone modification and DNA methylation changes (Ashbrook et al., 2018) shortly after the exposures. Furthermore, evaluation of this paradigm using a literature-derived, logic model of neuron-glia interactions indicated the potential for GWI to derive from an aberrant homeostatic neuroinflammatory profile (Craddock et al., 2018). Notably, these neuroinflammatory effects occur in the absence of significant peripheral inflammation (Michalovicz et al., 2019). While DFP alone has some minor proinflammatory effects in the liver and serum, these effects are largely suppressed by the prior CORT exposure, findings that differentiate them from the CORT priming of the DFP response in the brain and highlight the role of neuroinflammation in GWI. Interestingly, similar results were found in a clinical evaluation of neuroinflammation where veterans with GWI presented with an increased neuroinflammatory PET signal in the cortex in spite of there being no difference in plasma cytokine levels in comparison to healthy controls (Alshelh et al., 2020).

While no changes have been observed in astrocytes or microglia, the brain's primary immune cells, in the acute time points following exposure in this model (O'Callaghan et al., 2015), Belgrad et al. (2019) found that DFP alone had effects on oligodendrocytes, another glial cell type with immune function (Peferoen et al., 2014). In this study, DFP exposure decreased the number of mature and proliferating oligodendrocytes in the rat cortex and corpus callosum out to 21 days post-exposure while combined CORT and DFP exposure ameliorated these effects. However, this combined exposure resulted in an increase in myelin basic protein, a protein crucial to proper myelin structure, that may be indicative of demyelination or injury (Kristensson et al., 1986; Bartholdi and Schwab, 1998). As such, Naughton et al. (2018) found that their chronic DFP exposure paradigm resulted in disordered, de-compacted myelin sheaths. These exposures have also been translated into neuronal cell culture and shown to affect microtubule acetylation and axonal transport of mitochondria (Rao et al., 2017). In addition to these cellular and molecular level changes, high-order diffusion MRI of GWI rat brains demonstrated alterations in brain structure and connectivity concurrent with neuroinflammation that may be indicative of subtle structural changes in dendrites or glial processes (Koo et al., 2018). Taken at early time points following GWI exposure, these diffusion changes may capture the initiating conditions that have led to the more significant changes in brain structure reported by traditional MRI in veterans with GWI (Heaton et al., 2007; Chao et al., 2010, 2011, 2014, 2015; Chao and Zhang, 2018), and suggests neuroinflammation as an underlying cause.

While both sarin and DFP can significantly increase brain ACh levels by inhibiting AChE activity, the exacerbated neuroinflammation instigated by corticosterone priming of DFP exposure was associated with a reduction in ACh levels and mitigation of AChE inhibition in the brain (Locker et al., 2017; Miller et al., 2018). These studies provide strong evidence for a causative association between nerve agent and wartime stress exposure and GWI,

suggesting that these exposures have resulted in a persistent shift in how the neuroimmune system functions; ultimately, allowing for a chronic neuroinflammatory state that underlies the neurological and systemic issues experienced by the ill veterans. These results also propose a mechanism by which a condition of high physiological stress facilitated the circumvention of the anti-inflammatory cholinergic signaling pathway. Furthermore, the other studies discussed here (Phillips and Deshpande, 2016, 2018; Naughton et al., 2018) used low doses of DFP that did not produce acute cholinergic crisis. Though the potential for veterans with GWI to have been exposed to chemical weapons containing nerve agent has been highly controversial in the past, studies that have investigated these exposures at low doses, alone or in combination with the high physiological stress experienced in theater, have found compelling evidence for the involvement of nerve agent in the development of a chronic neuroimmune disorder underlying GWI. Moreover, the recent evidence indicating that wartime stress may have reduced the anticholinergic effects of these agents (Locker et al., 2017; Miller et al., 2018) suggests that other, non-cholinergic mechanisms are likely responsible for GWI (Terry, 2012; Naughton and Terry, 2018).

1.3. Chlorpyrifos (CPF) and dichlorvos (DDVP)

In addition to potential low-dose nerve agent exposure, frequent and pervasive pesticide usage also constituted a repeated, daily exposure in theater that was employed to help prevent vector-borne illnesses. According to the Environmental Exposure Report on Pesticides, soldiers were exposed to pesticides via treated uniforms and tents, flea collars, pest strips, fogging, and personal application with an estimated 41,000 having been overexposed (Winkenwerder, 2003); these exposures have been associated with GWI (Haley and Kurt, 1997; Golier et al., 2007; Golomb, 2008; Steele et al., 2012; White et al., 2016; Sullivan et al., 2018). Among these pesticides, veterans with GWI were exposed to the OP AChEIs, CPF and DDVP. Highlighting these exposures, a recent study of GW military pesticide applicators indicated a strong association between cognitive impairments and higher levels of exposure to DDVP pest strips (Sullivan et al., 2018). Unfortunately, to our knowledge, there has been no direct evaluation of GWI-related DDVP exposure in animal models, but DDVP exposure has the potential to produce neuroimmune responses such as microglial activation, increased inflammatory cytokine expression and neurodegeneration (Kaur et al., 2007; Binukumar et al., 2011). However, several studies have investigated the potential role of CPF exposure in GWI and found significant neurological effects (Ojo et al., 2014; Hernandez et al., 2015; Nutter et al., 2015; Cooper et al., 2016, 2018; Flunker et al., 2017; Locker et al., 2017; Miller et al., 2018). Among these studies, investigators have demonstrated that repeated exposure to CPF can cause persistent impairment of axonal transport (Hernandez et al., 2015), loss of synaptic integrity and neurogenesis in the hippocampus (Ojo et al., 2014), and decreased pain threshold when combined with PB, PER and DEET (Nutter et al., 2015; Cooper et al., 2018; Flunker et al., 2017; Cooper et al., 2018, 2018). While these results do not directly support the neuroimmune dysfunction hypothesis of GWI, conditions like neuropathic pain, impaired hippocampal neurogenesis, and axonal transport deficits have been associated with neuroinflammation (Ellis and Bennett, 2013; Walker et al., 2013; Errea et al., 2015; Valero et al., 2017). However, a few studies have directly evaluated neuroimmune-related consequences of CPF exposure. Specifically, it has been shown that exposure to the active metabolite of CPF, chlorpyrifos oxon (CPO; 8 mg/kg,

i.p.), results in exacerbated neuroinflammation in mice when combined with prior exogenous exposure to the stress hormone CORT as indicated by an increase of brain inflammatory cytokine mRNA across different brain areas (Locker et al., 2017; Miller et al., 2018). Chronic exposure to CPF alone or in combination with PB and the pesticide permethrin (PER) in mice for 10 days was found to cause an increase in GFAP, indicative of neuroinflammation-associated astrocyte activation; these results were brain region specific, with CPF alone increasing GFAP in motor cortex and hippocampus and CPF + PB + PER combined exposure causing astrogliosis in the piriform cortex and basolateral amygdala (Ojo et al., 2014). Furthermore, similar to the results seen with the sarin surrogate DFP, prior stressor exposure mitigated brain AChE inhibition and decreased ACh levels instigated by CPF exposure (Locker et al., 2017; Miller et al., 2018). While these studies highlight a role for OP AChEI pesticides in the neuroimmune dysfunction associated with GWI, more investigation is needed to understand the mechanisms by which they instigate illness (i.e. non-cholinergic pathways).

1.4. Pyridostigmine bromide (PB)

The requirement for soldiers deployed during the 1991 Gulf War to take prophylactic doses of PB in hopes of preventing serious complications from potential nerve agent exposures has made the drug a prime target for investigation in GWI. As a reversible AChEI with minimal permeability across the blood brain barrier (BBB), little risk was expected from prophylactic treatment with PB particularly considering the dire consequences of nerve agent exposure. Substantiating this notion is the prevalence of reversible AChEIs as pharmacological agents for the treatment of several illnesses, including myasthenia gravis, Alzheimer's disease, glaucoma, and others (Čolović et al., 2013). However, as early as 1997, epidemiological studies began to uncover an association between chemical exposures, including PB, and the emerging illness in a large population of veterans (Haley et al., 1997a; Haley et al., 1997b; Haley and Kurt, 1997; The Iowa Persian Gulf Study Group, 1997). Over the years, these initial studies have been expanded to reveal strong correlations between PB exposure alone or in combination with other chemicals and the various symptoms of GWI (Kurt, 1998; Nisenbaum et al., 2000; Schumm et al., 2001; Wolfe et al., 2002; Sullivan et al., 2003; Golomb, 2008; Research Advisory Committee on Gulf War Illnesses, 2008; Steele et al., 2012; Steele et al., 2015; White et al., 2016; Sullivan et al., 2018; Zundel et al., 2019). In particular, a few studies found a positive correlation between the number of PB pills taken and the severity of individual GWI cases (Wolfe et al., 2002; Lucas et al., 2007; Golomb, 2008). While most studies focused on evaluating GWI as the collection of its symptoms, a few studies have found specific associations between GWI, PB use, and neuromuscular dysfunction and suppressed cortisol responses (Golier et al., 2006, 2007), as well as gene-exposure interactions with butyrylcholinesterase genotypes (Steele et al., 2015) and increased risk of heart attack and diabetes (Zundel et al., 2019).

While these clinical findings have driven numerous preclinical studies to investigate the role of PB exposures in GWI, there has been minimal evidence to support its involvement in the underlying neuroimmune dysfunction associated with the illness. As such, when PB has been evaluated alone without exposure to any other mediating factors, the drug has minimal deleterious effects on mice or rats (Abou-Donia et al., 1996; Amourette et al., 2009;

Lamproglou et al., 2009; Barbier et al., 2009; Locker et al., 2017; Macht et al., 2018; Hernandez et al., 2019). While very few studies have looked directly at neuroinflammation as a result of PB exposure, those that have investigated this directly, found very minimal proinflammatory effects of the drug with an inclination towards anti-inflammatory outcomes (Locker et al., 2017; Hernandez et al., 2019). Moreover, while it has been suggested that stress may increase the permeability of the BBB allowing for PB to gain access to the brain (Friedman et al., 1996; Hanin, 1996; Shen, 1998; Shaikh et al., 2003), several studies investigating the possibility that wartime stress affected the brain accessibility of PB have found that multiple stressor methods do not increase BBB permeability, affect PB's reduction of brain ChE activity, nor elicit the elaboration of inflammatory markers in the brain or blood in animal models of GWI (Sinton et al., 2000; Song et al., 2002; Tian et al., 2002; Amourette et al., 2009; Locker et al., 2017; Macht et al., 2018). These results suggest that the relationship between PB exposure and GWI that has been supported by epidemiological studies is not straightforward but does not seem to support a role for PB alone in the neuroimmune dysfunction hypothesis.

1.4.1. PB in combination with other GW-relevant chemical exposures—As stated previously, veterans suffering with GWI had the potential to be exposed to many chemicals in theater. As such, a recent study of GW military pesticide applicators found a strong association between combined high pesticide and PB exposure and greater cognitive impairment along with higher rates of GWI (Sullivan et al., 2018). Other studies involving co-exposures to PB and sarin had mixed results. Abou-Donia et al. (2002) found that while PB offered some peripheral ChE protection it did not mitigate sarin-reduced brain AChE activity. Furthermore, while all exposure combinations (PB alone, sarin alone, PB + sarin) caused worsened sensorimotor impairments compared to controls, as assessed by grip strength and beam- and inclined plane-walking (Abou-Donia et al., 2002), the results were largely dependent on the dosage of sarin given and the amount of time following exposure. Specifically, combined exposure with PB was detrimental with higher dosages of sarin for the beam-walk score and degree at which slipping occurred on the inclined plane, but protective for the same tasks when combined with lower dosages of sarin. A separate study found that PB protected against short-term sarin-induced neurobehavioral impairment, as indicated by enhanced acoustic startle response and anxiety/decreased habituation in the open field test, while PB + sarin increased pain threshold at 16 weeks after exposure (Scremin et al., 2003). However, it is difficult to compare the results between these two studies as they used drastically different exposure models.

In addition, a significant number of exposure models have been developed that combine PB with other, non-AChEI, GW-relevant chemicals including: permethrin (PER) with or without stress (Abdullah et al., 2011; Zakirova et al., 2015; Alhasson et al., 2017; Nizamutdinov et al., 2018; Seth et al., 2018; Joshi et al., 2019); PER and DEET with or without stress (Abou-Donia et al., 1996; Abdel-Rahman et al., 2002; Abdullah et al., 2012; Parihar et al., 2013; Hattiangady et al., 2014; Megahed et al., 2015; Pierce et al., 2016; Shetty et al., 2017; Carerras et al., 2018; Petrescu et al., 2018; Madhu et al., 2019); PER and CPF with or without DEET (Ojo et al., 2014; Nutter et al., 2015; Cooper et al., 2016, 2018; Flunker et al., 2017). The prominence of these mixtures in GWI models, which largely revolve around

combined exposures with PER and DEET, stem from a recommendation in the report from the Institute of Medicine (US) Committee to Review the Health Consequences of Service During the Persian Gulf War (1995) and an initial study of these chemicals in a hen model (Abou-Donia et al., 1996). While some of these models have demonstrated neuroimmune-related effects, these models generally evaluate conditions only when all chemical exposures are combined and, therefore, the results are likely due to the other chemicals employed in these models rather than PB. However, one study presented by Cooper et al. (2018) found that inclusion of PB in their combination exposure with permethrin, chlorpyrifos, and DEET was requisite for the development of chronic pain and aberrant nociceptor signaling. Overall, the disparity in positive results between models that more directly investigate the role of PB in GWI and those that use combinations with other chemicals like sarin, PER, and/or CPF further highlights the likelihood that PB had a modulatory effect on the response to these other exposures in the initiation of GWI.

2. How did acetylcholinesterase inhibitor exposures cause the neuroimmune dysfunction associated with GWI?

While it has been suggested that there is a strong connection between AChEI exposures and the development of GWI and these chemicals have been demonstrated to have numerous cellular, biochemical, physiological, and neuropsychological effects, the direct mechanism by which exposure has resulted in this chronic illness has remained obscure. As summarized in this review, we suggest that the literature indicates that OPs that irreversibly inhibit AChE, like sarin and its surrogates, chlorpyrifos, and dichlorvos, seem to be the key exposure for the development of neuroimmune dysfunction in GWI. Under normal conditions, OP AChEIs exert their toxicological effects by organophosphorylating and inactivating the cholinesterase enzymes which produces acute toxicity. However, it is unclear whether exposed veterans with GWI exhibited any signs of acute AChEI intoxication. As far as potential sarin or cyclosarin exposure is concerned, the survey conducted of soldiers within the vicinity of the Kamisiyah detonation suggests that they did not experience signs of acute toxicity (Institute of Medicine (US) Committee on Health Effects Associated with Exposures During the Gulf War, 2000), suggestive of low-level exposure. As discussed in this review, a number of studies have recapitulated GWI in animal models using low doses of sarin, sarin surrogates, or pesticides. Similarly, it has been shown that under conditions of high physiological stress, the cholinergic toxicity of agents like DFP and CPO is suppressed as evidenced by a reduction in the percentage of enzyme inhibition and amelioration of the increase in brain ACh (Locker et al., 2017; Miller et al., 2018). We hypothesize that these conditions, e.g. low-level exposures alone or combined with high physiological stress, avoid the acute cholinergic toxicity of the OP AChEIs due to a dose-dependent interaction with the enzyme itself. In the case of low level exposure, the doses of AChEIs encountered may be below threshold for significant AChE inhibition. When considering the interaction of AChEI exposures and high physiological stress, studies have shown that stress or CORT exposures can increase AChE activity (Oriaku and Soliman, 1986; Fatranska et al., 1987; Tsakiris and Kontopoulos, 1992); thus, altering the activity of AChE in the tissue has the potential to compensate for the inhibition instigated by AChEI exposure by normalizing ACh levels. Therefore, in both of these conditions, exposure to OP AChEIs would have minimal

functional impact on cholinergic signaling and not trigger the prototypical cholinergic anti-inflammatory signaling pathway (Pavlov et al., 2003; Pavlov and Tracey, 2005); facilitating the significant neurological results demonstrated following OP AChEI exposure, including signs of neuroimmune dysfunction, such as increased expression of neuroinflammatory cytokines and astrogliosis (Ojo et al., 2014; Locker et al., 2017; Miller et al., 2018). Thus, we suggest that GWI may be the result of aberrant neuroimmune signaling that is instigated by the organophosphorylation of non-cholinesterase targets by the OP AChEIs, a hypothesis that has been previously suggested (Grigoryan et al., 2008, 2009; Terry et al., 2012; Naughton and Terry, 2018) and warrants further investigation in relationship to neuroinflammatory signaling.

3. Conclusions

As stated throughout this review, GWI is believed to be a result of a combination of exposures/conditions that were experienced by soldiers during deployment. In the years that have followed the 1991 Gulf War, research has suggested that AChEIs are chief among these exposures as culprits in the development of GWI, particularly when combined with stressors to mimic the extreme conditions experienced by soldiers during deployment. Overall, the irreversible, OP AChEIs, both nerve agents and pesticides, are more likely to have played a primary role in the development of GWI as they show a strong correlation with the neuroimmune dysfunction suspected to be the underlying cause of illness among these veterans. While the administration of PB pills has long been suspect in the development of GWI, our review of the literature has suggested that the correlation between PB and GWI is not strongly supported by preclinical investigations using animal models of exposure; thus, the role of PB in GWI remains unclear. In spite of their common cholinergic functions, we strongly suspect that GWI is the result of the actions of OP AChEI exposures on non-cholinergic targets (Grigoryan et al., 2008, 2009; Terry et al., 2012; Locker et al., 2017; Miller et al., 2018; Naughton and Terry, 2018), namely organophosphorylation of proteins that are crucial to neuroinflammatory signaling. Long-term low dose inhibition of AChE by these compounds and other contributing factors, such as physiological stress and other chemical exposures, may have played a role as well to facilitate the development of a chronic neuroimmune disorder (Fig. 1). By the continued investigation into potential mechanisms underlying GWI pathobiology, we may be able to uncover therapeutic targets that can be modulated to more successfully treat GWI symptoms and/or reverse the underlying aberrant neuroimmune function associated with this disorder.

Acknowledgements

This work was supported by a Department of Defense, Congressionally Directed Medical Research Program: Gulf War Illness award (W81XWH-13-2-0072). The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention. This work was supported by the Assistant Secretary of Defense for Health Affairs, through the Gulf War Illness Research Program. Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the Department of Defense.

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HIGHLIGHTS

- Gulf War Illness (GWI) is associated with underlying neuroimmune dysfunction.
- Veterans with GWI were exposed to several acetylcholinesterase inhibitors (AChEIs).
- Organophosphate AChEIs, nerve agent and pesticides, can instigate neuroinflammation.
- While associated with GWI, pyridostigmine does not cause neuroinflammation.
- Lack of acute toxicity suggests GWI results from non-cholinergic actions of AChEIs.

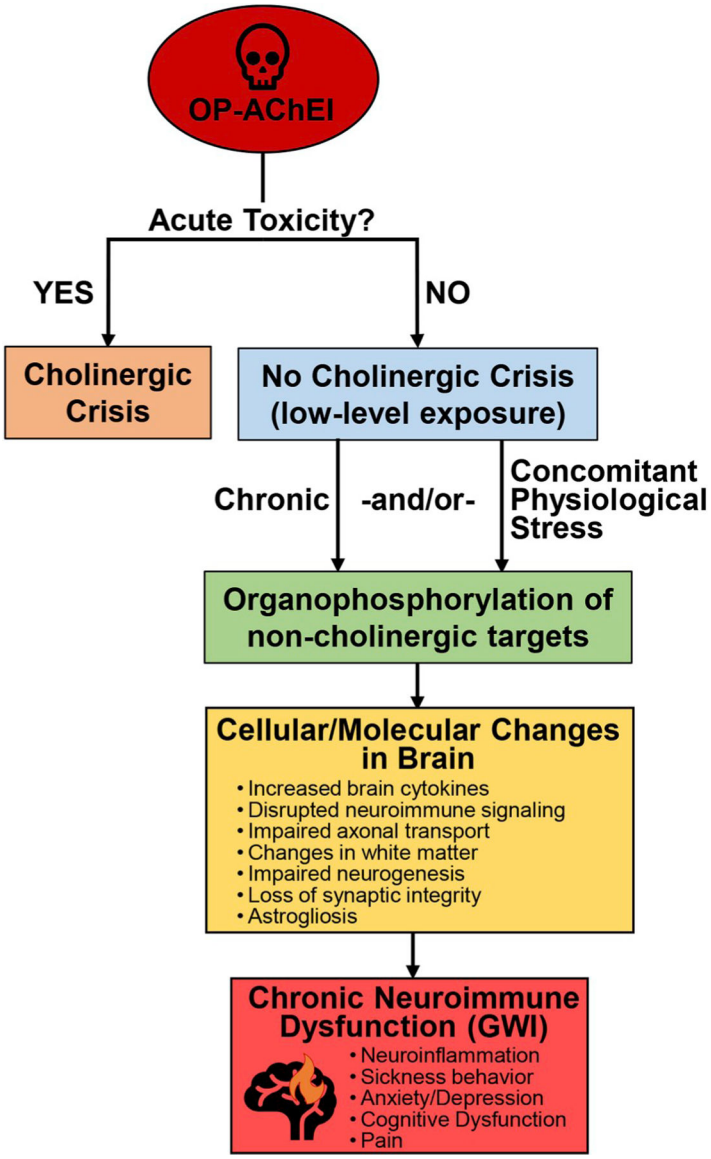

Fig. 1. Mechanism of acetylcholinesterase inhibitor-induced neuroimmune dysfunction in Gulf War Illness.

Acetylcholinesterase inhibitors (AChEIs), in particular the organophosphate (OP) chemicals, can instigate illness in two ways: (1) acute toxicity that results in cholinergic crisis (salivation, lacrimation, urination, defecation, gastrointestinal upset, emesis, miosis [SLUDGEM]; seizures) and carries a higher risk of mortality; (2) long-term illness in the absence of an acute cholinergic crisis. The latter condition is proposed to be the result of chronic low-level AChEI exposure with or without concurrent exposure to physiological stress. The myriad of cellular and molecular effects that have been demonstrated in the brain as a result of these exposures are hypothesized to be the consequences of organophosphorylation of non-cholinergic targets, e.g. neuroinflammatory signaling mediators. Ultimately, these effects culminate into a state of chronic neuroimmune dysfunction, the underlying cause of Gulf War Illness (GWI).

brain sciences

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TLR Antagonism by Sparstolonin B Alters Microbial Signature and Modulates Gastrointestinal and Neuronal Inflammation in Gulf War Illness Preclinical Model

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Received: 14 July 2020; Accepted: 5 August 2020; Published: 8 August 2020

Abstract: The 1991 Persian Gulf War veterans presented a myriad of symptoms that ranged from chronic pain, fatigue, gastrointestinal disturbances, and cognitive deficits. Currently, no therapeutic regimen exists to treat the plethora of chronic symptoms though newer pharmacological targets such as microbiome have been identified recently. Toll-like receptor 4 (TLR4) antagonism in systemic inflammatory diseases have been tried before with limited success, but strategies with broad-spectrum TLR4 antagonists and their ability to modulate the host-microbiome have been elusive. Using a mouse model of Gulf War Illness, we show that a nutraceutical, derived from a Chinese herb Sparstolonin B (SsnB) presented a unique microbiome signature with an increased abundance of butyrogenic bacteria. SsnB administration restored a normal tight junction protein profile with an increase in Occludin and a parallel decrease in Claudin 2 and inflammatory mediators high mobility group box 1 (HMGB1), interleukin-1β (IL-1β), and interleukin-6 (IL-6) in the distal intestine. SsnB also decreased neuronal inflammation by decreasing IL-1β and HMGB1, while increasing brain-derived neurotrophic factor (BDNF), with a parallel decrease in astrocyte activation in vitro. Mechanistically, SsnB inhibited the binding of HMGB1 and myeloid differentiation primary response protein (MyD88) to TLR4 in the intestine, thus attenuating TLR4 downstream signaling. Studies also showed that SsnB was effective in suppressing TLR4-induced nod-like receptor protein 3 (NLRP3) inflammasome activation, a prominent inflammatory disease pathway. SsnB significantly decreased astrocyte activation by decreasing colocalization of glial fibrillary acid protein (GFAP) and S100 calcium-binding protein B (S100B), a crucial event in neuronal inflammation. Inactivation of SsnB by treating the parent molecule by acetate reversed the deactivation of NLRP3 inflammasome and astrocytes in vitro, suggesting that SsnB molecular motifs may be responsible for its anti-inflammatory activity.

Keywords: dysbiosis; butyrogenic bacteria; neuroinflammation; nutraceutical; HMGB1; BDNF; astrocytes

1. Introduction

Gulf War Illness (GWI) is a chronic, multi-symptomatic disease condition that is known to affect nearly one-third of the veterans who were deployed in the Persian Gulf War of 1990–1991 [1,2]. The symptoms include chronic fatigue, musculoskeletal pain, cognitive deficiencies, respiratory and gastrointestinal disturbances [3]. Exposure to a wide array of toxic chemicals individually or in combination, in the war theatre, was attributed to being the main reason for GWI [3]

Studies show that administration of pyridostigmine bromide (PB) and permethrin (Per) led to gastrointestinal and neurological disturbances in GWI mouse models [4,5]. Our group was the first to report that the GWI mouse model exhibited significant alteration of the gut microbiome, leading to gut leaching, increased endotoxemia, and Toll-like receptor 4 (TLR4) activation which established a mechanistic connection between gastrointestinal and neuroinflammation [6]. We have further confirmed that the administration of the Gulf War (GW) chemicals in mice led to the activation of enteric glial cells through the Toll-like receptor (TLR) pathway [7]. Though there has been extensive research on the underlying pathobiology of GWI over the past decade, yet there are few reported therapeutic approaches specific for GWI that target the Toll-like receptors [8–11].

TLRs play an important role in innate and adaptive immunity [11]. They are expressed in the intestinal epithelial cells (IEC) and detect pathogen-associated molecular patterns (PAMPs) similar to bacterial lipopolysaccharides (LPS) from invading pathogens. TLRs also recognize endogenous signals such as damage-associated molecular patterns (DAMPs), e.g., HMGB1, and recruit adaptor molecules such as myeloid differentiation primary response protein (MyD88) molecules to trigger immunological responses. Activation of TLR4 leads to pro-inflammatory responses causing neuronal injury and secretion of neurotoxic compounds in Alzheimer's disease (AD) and Parkinson's disease (PD) [12,13]. We have already established that an increased TLR4 activation is a major cause of the gastrointestinal and neuro-inflammation in GWI [6,12]. Therefore, it led us to investigate the therapeutic effects of TLR2-4 antagonists in our current study [6].

Due to the absence of clinically approved TLR antagonists, we used a potentially established TLR4 antagonist in preclinical studies, Sparstolonin B (SsnB), a natural compound that was isolated from the Chinese herb *Sparganium stoloniferum* [13]. The structural analysis identified SsnB to be a polyphenol having similarities with isocoumarin and xanthone, which attributed to its anti-inflammatory effects. It is reported that SsnB inhibits the TLR4 activation by blocking the binding of TLR4 to MyD88, thereby suppressing/decreasing the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation [14–16]. SsnB has also been reported to improve outcomes in intracerebral hemorrhage and neuropathic pain in a mice model through a TLR4 dependent manner [17,18].

With the above evidence, we hypothesized that the administration of SsnB in the GWI mouse model using PB and Per would significantly modulate gut microbiome, aid in the recovery of GW chemical-induced gut dysbiosis, attenuate gut leaching and expression of DAMPS by antagonizing TLR4 induced inflammation thus improving gut and brain health. In this study, we used an in vivo and in vitro approach to demonstrate the role of SsnB in downregulating TLR4 pathway mediated inflammation in a mouse model of GWI.

2. Material and Methods

Per, PB, and Sparstolonin B (SsnB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-Claudin 2, anti-Occludin, anti-interleukin-6 (IL-6), anti-nod-like receptor protein 3 (NLRP3), anti-Caspase 1, and anti-β-actin were purchased from Abcam (Cambridge, MA USA). Anti-Toll-like receptor 4 (TLR4), anti-S100 calcium binding protein B (S100B), anti-brain derived neurotrophic factor

(BDNF), and anti-interleukin-1β (IL-1β) antibodies were purchased from Santacruz Biotechnology (Dallas, TX, USA). Anti-myeloid differentiation primary response protein (MyD88), anti-high mobility group box 1 (HMGB1), anti-apoptosis associated speck-like protein containing a caspase recruitment domain (ASC2), and anti-glial fibrillary acid protein (GFAP) antibodies were purchased from Abclonal Technology (Woburn, MA, USA). Anti-nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB p65) and anti-phospho NF-κB p65 were purchased from Cell Signaling Technology (Danvers, MA, USA). Species-specific biotinylated conjugated secondary antibody and Streptavidin-horse radish peroxidase (HRP) (Vectastain Elite ABC kit) were purchased from Vector laboratories (Burlingame, CA, USA). Fluorescence conjugated Alexa Fluor secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Recombinant mouse HMGB1 was purchased from BioLegend (San Diego, CA, USA). All other chemicals used in this study unless specified were purchased from Sigma. Paraffin embedding of tissues and sectioning on slides were done by AML Laboratories (Jacksonville, FL, USA).

2.1. Animals

Adult wild-type male (C57BL/6J) mice of age ten weeks were purchased from Jackson Laboratories (Bar Harbour, ME). The implementation of mice was done in accordance with National Institutes of Health (NIH) guidelines for human care and use of laboratory animals and local IACUC standards. All procedures were approved by the University of South Carolina at Columbia, SC. In accordance with current regulations and guidelines, the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina has reviewed and approved an action on Animal Use Proposal reference number 101345 (protocol number: 2419-101345-072318) with approval date 11/8/2019. The mouse was housed individually and fed with a chow diet at 22–24 °C with 12 h light/12 h dark cycle. The mice were sacrificed after animal experiments had been completed in seven days. The serum was prepared from freshly obtained blood from mice by cardiac puncture immediately after anesthesia. The serum was preserved at −80 °C until further analysis was performed. The distal part of small intestine and frontal cortex was collected from dissected mice and fixed in 10% neutral buffered formaldehyde or Bouin's solution (Sigma-Aldrich, St. Louis, MO, USA), respectively and further processed for immunostaining. Fecal pellets were collected from the colon and stored in −80 °C for microbiome analysis.

2.2. Rodent Model of Gulf War Illness

Mice were exposed to Gulf War (GW) chemicals pyridostigmine bromide (PB) and permethrin (Per) following established rodent models of Gulf War Illness (GWI) with modifications (7). The acclimatized mice were randomly divided into four groups. The first group was a control (CONT) (n = 3) and dosed with vehicle (0.6% dimethyl sulphoxide (DMSO) in a phosphate buffered saline (PBS)). The second group (GW) (n = 3) received GW chemicals permethrin (200 mg/kg diluted in DMSO) and pyridostigmine bromide (2 mg/kg diluted in PBS) by oral gavage. The third group (GW+SsnB) (n = 3) were treated with PB and Per along with SsnB (3 mg/kg diluted in DMSO) administered intraperitoneally. The fourth group of mice (SsnB) received SsnB through an intraperitoneal route, which served as a control for the GW + SsnB group. GW chemicals and SsnB were dosed every alternate day for a week receiving a total of four doses.

2.3. Cell Culture

2.3.1. Mouse Primary Intestinal Epithelial Cell Culture

Mouse primary intestinal epithelial cells (C576051) were purchased from Cell biologics (Chicago, IL, USA). The cells were maintained according to guidelines provided by Cell biologics. Cells were plated in 12- and 6-well tissue culture plates and allowed to reach 70% confluency in growth. Serum starvation was done using a 1% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM) for 18 h and then treated with chemicals. The cells were treated with a vehicle control (0.05% DMSO),

mouse recombinant HMGB1 (50 ng/mL), SsnB (10 and 100 µg/mL), and inactivated SsnB (SsnBi) (10 and 100 µg/mL). Inactivation was done by adding an equal concentration of acetate with SsnB [19]. All treatments were for 24 h. After 24 h, the cells were harvested for immunofluorescence staining and protein extraction for Western blot.

2.3.2. Mouse Primary Astrocyte Cell Culture

Primary mouse astrocytes C8-D1A [Astrocyte type I clone] (ATCC CRL-2541) were obtained from ATCC (Manassas, VA, USA). The cells were maintained in DMEM supplemented with 10% FBS. The cells were plated in 12- and 6-well tissue culture plates and growth were allowed until 70% confluency. The cells were sera starved using a 1% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM) for 18 h and then treated with chemicals. The cells were treated with a vehicle control (0.05% DMSO), mouse recombinant HMGB1 (50 ng/mL), SsnB (10 and 100 µg/mL), and inactivated SsnB (SsnBi) (10 and 100 µg/mL). All treatments were for 24 h. After 24 h the cells were harvested for immunofluorescence staining.

2.4. Microbiome Analysis

Microbiome analysis was performed using the fecal pellets collected from the mouse after euthanization. Briefly, 100 mg of fecal pellets were used to isolate genomic DNA using the QIamp DNA stool mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Amplification of a 16S rRNA V3-V4 hypervariable region added with an Illumina adapter overhang nucleotide sequence to create a DNA library. The Illumina Miseq platform (San Diego, CA, USA) was used for sequencing. The reads from sequencing were further analyzed by Nephele, an analysis tool by the National Institute of Allergy and Infectious Diseases (NIAID) Office of Cyber Infrastructure and Computational Biology (OCICB) in Bethesda, MD, USA. QIIME FASTQ paired-end with chimera removal, open reference, and the SILVA rRNA database project was used for microbial profiling.

2.5. Laboratory Methods

2.5.1. Immunohistochemistry

The distal part of a mouse small intestine and frontal cortex tissues was paraffin embedded and prepared according to the standard protocols and 5 µm thick sections were made. Deparaffinization of the sections were done using the standard protocol. Epitope retrieval of the tissue sections was performed using an epitope retrieval solution and steamer (IHC World, Woodstock, MD, USA). Peroxidase blocking was done using 3% hydrogen peroxide (H_2O_2). Blocking was done with 10% serum followed by overnight incubation at 4 °C with primary antibodies against HMGB1, IL-6, IL-1β, and BDNF at 1:200 dilutions. Species specific biotin conjugated secondary antibodies and streptavidin conjugated horseradish peroxidase (HRP) at 1:500 dilutions were used for immunohistochemistry. 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich, St Louis, USA) was used as a chromogenic substrate. Counterstaining was done using Mayer's hematoxylin solution (Sigma-Aldrich). The stained sections were mounted with Aqua Mount (Lerner Laboratories, Kalamazoo, MI, USA). Sections were observed using the Olympus BX43 and BX63 microscope (Olympus, USA). Morphometric analysis of the images was done using the Cellsens software from Olympus (Center Valley, PA, USA).

2.5.2. Immunofluorescence Staining

Paraffin embedded distal part of mouse small intestine and frontal cortex tissues were deparaffinized using a standard protocol. Epitope retrieval of the tissue sections was performed using an epitope retrieval solution and steamer (IHC World, Woodstock, MD, USA). 10% serum was used for blocking followed by overnight incubation at 4 °C with primary antibodies against Occludin, Claudin 2, TLR4, HMGB1, MyD88, NLRP3, ASC2, Caspase 1, GFAP, and S100B were used at 1:200 dilutions. Species-specific secondary antibodies conjugated with Alexa Fluor (633 red and 488 green) were used at

1:250 dilutions. Mounting of the stained sections was done using a Prolong Diamond antifade reagent with DAPI. Tissue sections were observed under the Olympus BX43 and BX63 fluorescence microscope (Olympus, USA). Morphometric analysis of the images was done using the Cellsens software from Olympus (Center Valley, PA, USA).

2.6. Western Blot

Protein samples were prepared from tissues (distal part of the small intestine and frontal cortex) and cells using a RIPA lysis buffer and quantification were done by the bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, IL, USA). Thirty μg of protein from tissue and cell lysates were resolved on Novex 4–12% bis-tris gradient gel and subjected to standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were then transferred to the nitrocellulose membrane using the Trans-Blot Turbo transfer system (Bio-Rad, CA, USA). The membrane was stained with Ponceau S followed by blocking in 5% bovine serum albumin (BSA). Incubation with primary antibodies (1:1000 dilution) was done overnight at 4 °C. Species specific anti-IgG secondary antibody conjugated with HRP (1:5000 dilution) was used to tag primary antibody. Blots were developed using ECL Western blotting substrate. The blots were imaged using G: Box Chemi XX6 and densitometric analysis was performed using ImageJ software.

2.7. Serum Enzyme linked Immunosorbent Assay (ELISA)

Serum HMGB1 level was estimated with sera from CONT, GW, GW + SsnB, and SsnB mice groups using the ELISA kit from Abclonal Technology (Woburn, MA, USA) following the manufacturer's protocol.

2.8. Statistical Analysis

The in vivo and in vitro experiments were repeated three times and the data were pooled together. We used unpaired t-test and analysis of variance (ANOVA) for statistical analysis followed by Bonferroni post-hoc corrections for comparing between the groups. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. SsnB Administration in the GWI Mouse Model Presented a Unique Microbiome Pattern of Higher Abundance of Butyrogenic Bacteria and Prevented Alteration of Tight Junction Protein Levels Claudin 2 and Occludin

To study whether administration of SsnB in the GW mouse model could restore GW chemical (PB and Per) induced microbial dysbiosis, we analyzed mouse fecal pellets using 16S V3-V4 sequencing to study the relative abundance of the microbial load (Figure 1A). The relative abundance of each genus between the four mouse groups are depicted as a percentage, and the overall relative abundance across all the genus identified in microbial analysis is depicted as numerical values. Interestingly, we found that the Firmicutes-Bacteroidetes ratio was increased in GW + SsnB (72%:23%) group compared to the GW chemical exposed group (60%:40%) (Figure S1). At the genus level, we found that the abundance of beneficial microbes belonging to the Firmicutes phyla were increased in the GWI + SsnB group as compared to the GW group. Relative abundance calculated from individual genus OTU percentages of butyrogenic bacteria such as *Lactobacillus*, *Ruminococcaceae UCG014*, *Ruminococcaceae UCG009*, *Lachnospiraceae UCG001*, and *Roseburia* which belong to phyla Firmicutes were increased in the GW + SsnB group compared to the GW group. The relative abundance of *Blautia*, however, which also belongs to the butyrogenic bacteria group, was decreased in GW + SsnB compared to the GW group. SsnB administration also increased the relative abundance of genus *Turicibacter* of phylum Firmicutes. The relative abundance of *Enterorhabdus*, a group of bacteria associated with gastrointestinal inflammation and colitis, was found to decrease in the GW + SsnB group compared to GW group

of mice [20]. Notably, SsnB administration showed a 16-fold decrease in the relative abundance of *Akkermansia* of phylum Verucomicrobia in GW + SsnB compared to GW group. *Akkermansia* is an inherent gut microbe responsible for maintaining gut barrier function [21].

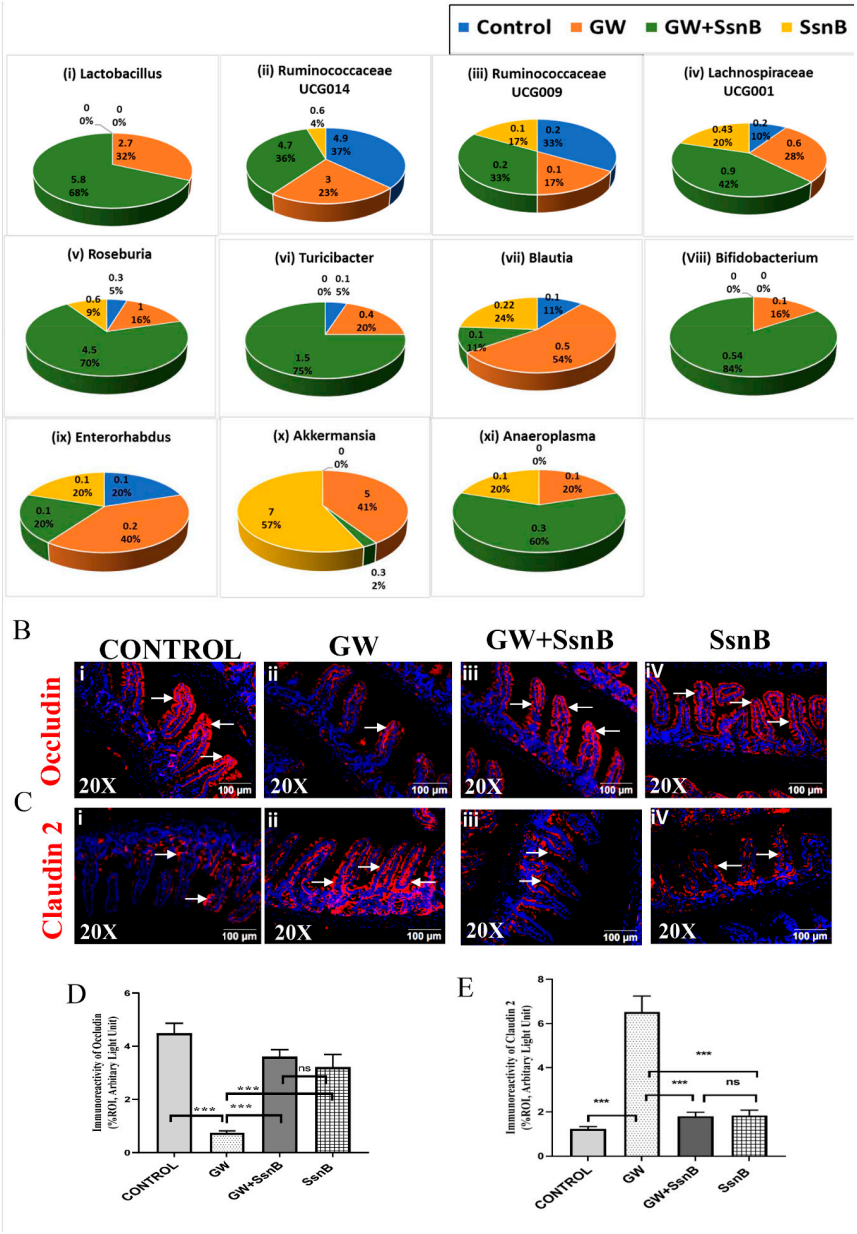

Figure 1. Sparstolonin B (SsnB) restores Gulf War (GW) chemical-induced gut microbial dysbiosis and restores gut leaching. Graphical representation of relative abundance of the microbiome at the genus level that was significantly altered (Figure 1A). The groups compared are CONT (n = 3) (wild-type mice which were treated with vehicle), GW (n = 3) (GW chemical exposed group), GW + SsnB (n = 3) (group exposed with GW chemicals and SsnB), and SsnB (n = 3) (group treated with SsnB only). Representative images of the expression of tight junction proteins Occludin (Figure 1B) and Claudin 2 (Figure 1C) were studied by immunofluorescence microscopy. All small intestine sections were stained with a red fluorescent secondary antibody for tight junction protein and were counterstained with DAPI (blue). Immunoreactivity was observed around the epithelial cells of the villi marked by white arrows. Images were taken in 20× magnification. Bar graphs depicting morphometric analysis of Occludin and Claudin 2 immunoreactivity are represented as mean ± standard deviation (SD) of the %ROI (mean value calculated from three separate fields of a section of small intestine) (Figure 1D,E). Significance was analyzed by unpaired T-test where ** $p < 0.01$, *** $p < 0.001$.

Tight junction proteins are transmembrane proteins that maintain the integrity of the gut barrier against foreign particles and invading pathogens [22]. In the present study, we wanted to examine whether administration of SsnB restored the altered tight junction protein expression due to GW chemical exposure by immunostaining for Claudin 2 and Occludin followed by immunofluorescence microscopy. Results showed a nearly significant increased expression of Claudin 2 and decreased expression of Occludin in the GW group compared to control (Figure 1Ci,ii,Bi,ii,D,E). SsnB administration to GW chemical exposed mice group showed significantly increased expression of Occludin ($p < 0.01$) and significant decreased expression of Claudin 2 ($p < 0.01$) compared to the GW mouse group (Figure 1Biii,iv,Ciii,iv,D,E).

3.2. SsnB Attenuates GW Chemical-Induced Expression of Pro-Inflammatory Cytokines and Damage-Associated Molecular Pattern HMGB1

In the present study, we wanted to study if the administration of SsnB could decrease the expression of the pro-inflammatory markers such as IL-1β, IL-6, and DAMPs like HMGB1 that has been shown by us and others as markers for GI inflammation. We probed sections of small intestine with antibodies for HMGB1, IL-1β, and IL-6 by immunohistochemistry to study the immunoreactivity. The expression of HMGB1, IL-1β, and IL-6 was found to be significantly increased in the GW group compared to the control ($p < 0.01$) as observed by the immunoreactivity in the villi of the small intestine (Figure 2Ai,ii,Bi,ii,Ci,ii,D,E,F). However, in the GW + SsnB group, we found that the expression of HMGB1, IL-1β, and IL-6 was significantly decreased ($p < 0.01$) as compared to the GW group (Figure 2Aiii,Biii,Ciii,D,E,F). In a group treated with SsnB only, a similar decrease in expression of pro-inflammatory cytokines was observed ($p < 0.01$) (Figure 2Aiv,Biv,Civ,D,E,F). These results strongly suggest the anti-inflammatory role of SsnB. Results showed a significant increased level of serum HMGB1 in GW chemical exposed mice group ($p < 0.01$) compared to the control (Figure 2G). However, coexposure of SsnB and GW chemicals in mice significantly decreased the level of serum HMGB1 as compared to the GW group ($p < 0.01$) (Figure 2G).

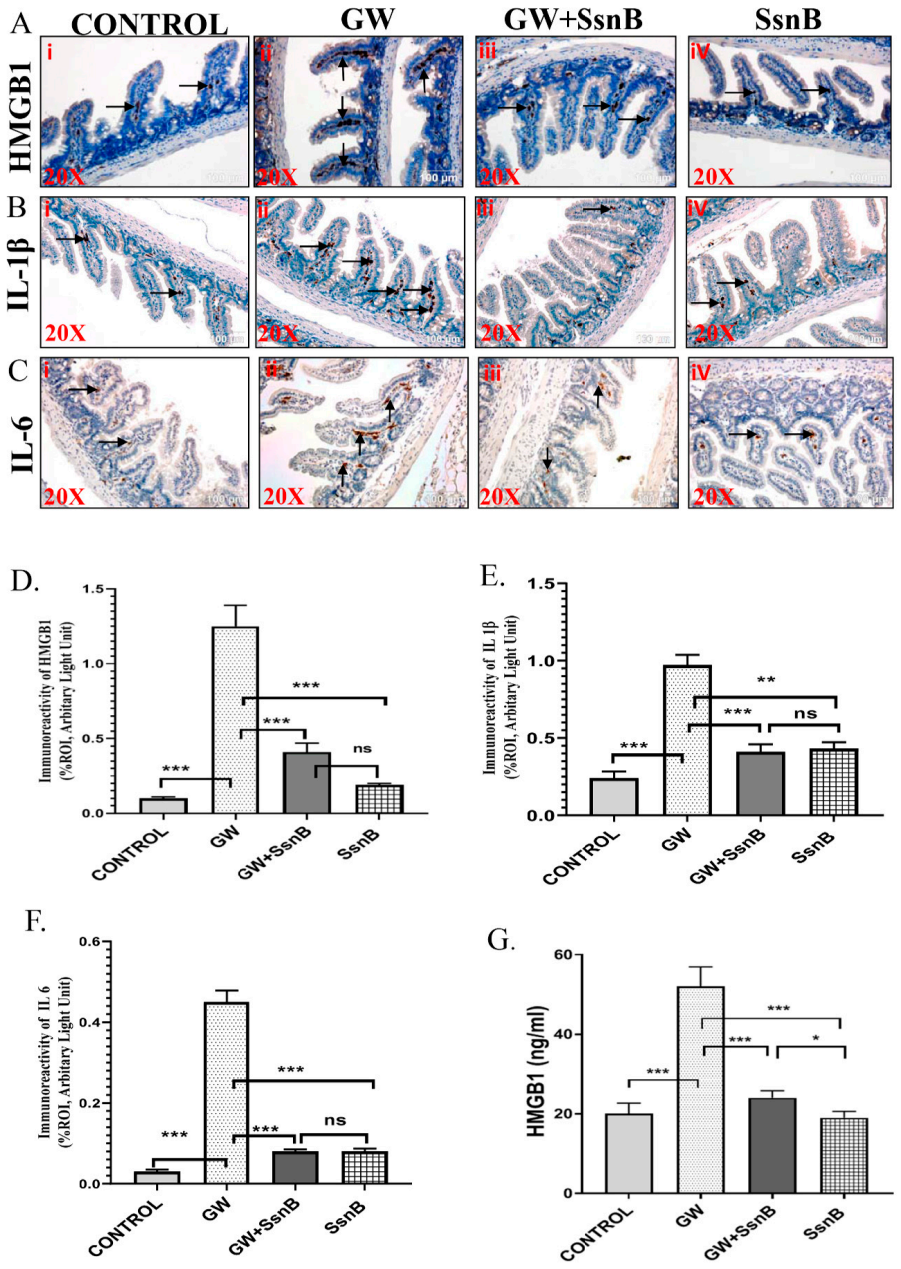

Figure 2. SsnB administration attenuates GW chemical-induced expression of pro-inflammatory cytokines and damage-associated molecular pattern high mobility group box 1 (HMGB1). Representative immunohistochemistry images of small intestine showing immunoreactivity (reactivity was majorly observed in the villi marked with black arrows) for HMGB1 (Figure 2A), IL-1β (Figure 2B), and IL-6 (Figure 2C) in CONT (n = 3) (wild-type mice which were treated with vehicle), GW (n = 3) (GW chemical exposed group), GW + SsnB (n = 3) (group exposed with GW chemicals and SsnB), and SsnB (n = 3) (group treated with SsnB only). Images were taken in 20× magnification. Bar graphs depicting morphometric analysis of HMGB1 (Figure 2D), IL-1β (Figure 2E), and IL-6 (Figure 2F) immunoreactivity are represented as mean ± SD of the %ROI (mean value calculated from three separate fields of a section of the small intestine). Significance was analyzed by unpaired T-test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Bar graph depicting the serum HMGB1 level at ng/mL (Figure 2G). Significance was analyzed by unpaired T-test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3. SsnB Attenuates Gulf War Chemical-Induced TLR4 Activation in the Small Intestine of the GWI Mouse Model

In our previous studies in a GWI mouse model, we have found that gut leaching and increased expression of pro-inflammatory cytokines is mechanistically related to TLR4 activation in the small intestine (6). Here, we found that administration of GW chemicals significantly increased expression of HMGB1. HMGB1 is known to be a potent TLR4 ligand that triggers pro-inflammatory signals [23]. Hence, we used immunofluorescence microscopy to study the colocalization of HMGB1/TLR4 by dual labeling HMGB1 (green) and TLR4 (red). Results showed that colocalization events (yellow color) of HMGB1/TLR4 in the epithelial cells surrounding the villi (marked by white circles) was increased in the GW chemical exposed group (GW) compared to the control ($p < 0.01$) (Figure 3Ai,ii,C). Moreover, the colocalization events were significantly decreased in the GW + SsnB treated group compared to the GW group ($p < 0.01$) (Figure 3Aiii,iv,C).

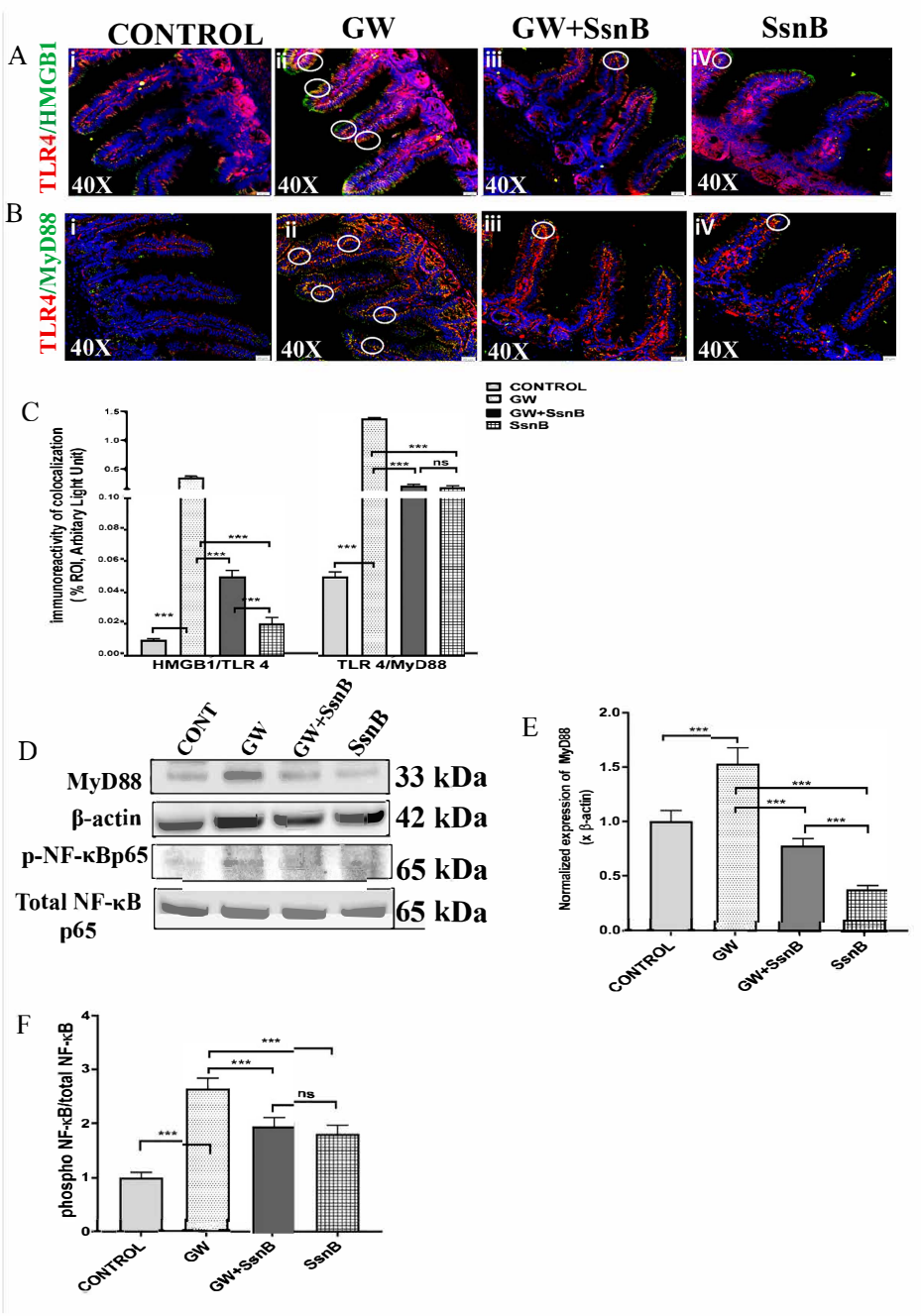

Figure 3. SsnB attenuates Gulf War chemical-induced HMGB1 mediated Toll-like receptor 4 (TLR4) activation in the small intestine of the GWI mouse model. Damage-associated molecular patterns (DAMPs) released due to gut dysbiosis further activates inflammatory signaling pathways through TLR4 receptors. Colocalization study of TLR4 (red)/HMGB1 (green) (Figure 3A) and TLR4 (red)/MyD88 (green) (Figure 3B) was performed using immunofluorescence microscopy in small intestine sections of CONT (n = 3) (wild-type mice which were treated with vehicle), GW (n = 3) (GW chemical exposed group), GW + SsnB (n =3) (group exposed with GW chemicals and SsnB), and SsnB (n = 3) (group treated with SsnB only). Colocalizations were observed in the epithelial cells of the villi as yellow dots and were marked in the representative images by white circles. Images were taken at 40× magnification. Bar graphs depicting morphometric analysis of TLR4 /HMGB1 and TLR4/MyD88 (Figure 3C) colocalizations are represented as mean ± SD of the %ROI (mean value calculated from three separate fields of a section of small intestine). Significance was analyzed by unpaired T-test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Immunoblot analysis using small intestine tissue lysates was performed to study the role of SsnB in the expression of TLR4 signaling molecules MyD88 and NF-κB (total p65 and phosphorylated p65) (Figure 3D). Bar graph depicting densitometric analysis of MyD88 normalized with β-actin represented mean ± SD (n = 3) (Figure 3E). Bar graph depicting densitometric analysis of phosphorylated p65 was normalized with total p65 (Figure 3F). Significance was analyzed by unpaired T-test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

It is reported that SsnB attenuates TLR4 activation by attenuating the association of adaptor protein MyD88 with TLR4 and consequently the phosphorylation of NF-κB (13). We performed immunofluorescence microscopy to study the interaction of TLR4 with MyD88 through colocalization studies by dual-labeling mouse small intestine sections with TLR4 (red) and MyD88 (green) antibodies. A significant increase in TLR4/MyD88 colocalization events (yellow) was observed (marked in a white circle) in the GW chemical treated group (GW) compared to the control group (CONT) ($p < 0.01$) (Figure 3Bi,ii,C). The colocalization of TLR4/MyD88 was significantly decreased in GW + SsnB and SsnB only groups ($p < 0.01$) (Figure 3Biii,iv,C). Western blot was performed with small intestine tissue lysates from all the groups for protein expression studies. The Western blot results corroborated with our immunofluorescence results, and it showed a significant increase in the expression of MyD88 in GW groups compared to the control. Notably, the expression of MyD88 was markedly decreased in the GW + SsnB group ($p < 0.01$) (Figure 3D,E). Further, we performed Western blot to study the role of SsnB in decreasing the activation of NF-κB. We found that the expression of phosphorylated NF-κB p65 (on comparing with total NF-κB p65) was significantly increased in the GW group compared to the control. However, NF-κB activation was significantly decreased in the GW + SsnB group ($p < 0.01$) (Figure 3D,F).

3.4. Priming with SsnB Attenuates GW Chemical-Induced NLRP3 Inflammasome Activation in the GWI Mouse Model

The secretion of pro-inflammatory cytokines such as IL-1β occurs through the activation of cytosolic pattern recognition receptors (PRRs) called inflammasomes [24]. NLRP3 is one such inflammasome that can be activated by DAMPs like HMGB1 [25]. In order to study NLRP3 activation in the GW chemical exposed group and the potential of SsnB in downregulating its activation, immunofluorescence microscopy was used. Briefly, we dual-labeled mouse small intestine section with NLRP3 (red) and its adaptor protein ASC2 (green) antibodies. Significantly increased colocalization events of NLRP3-ASC2 was observed (observed as yellow dots on the epithelial cells in villi, marked by white circle) in the GW group compared to the control group ($p < 0.01$) (Figure 4Ai,ii,C) confirming NLRP3 inflammasome activation in the intestine of GW mouse groups. Consequently, the colocalization events were considerably decreased in the GW + SsnB and group treated with SsnB only ($p < 0.01$) (Figure 4Aiii,iv,C). To further confirm the decrease of NLRP3 inflammasome activation by SsnB, co-localization events of NLRP3 (red), and Caspase 1 (green) was studied in the intestine sections.

Results showed significantly decreased colocalization events of NLRP3/Caspase 1 in the GW + SsnB group and groups treated with SsnB only as compared with the GW group ($p < 0.01$) (Figure 4B,C).

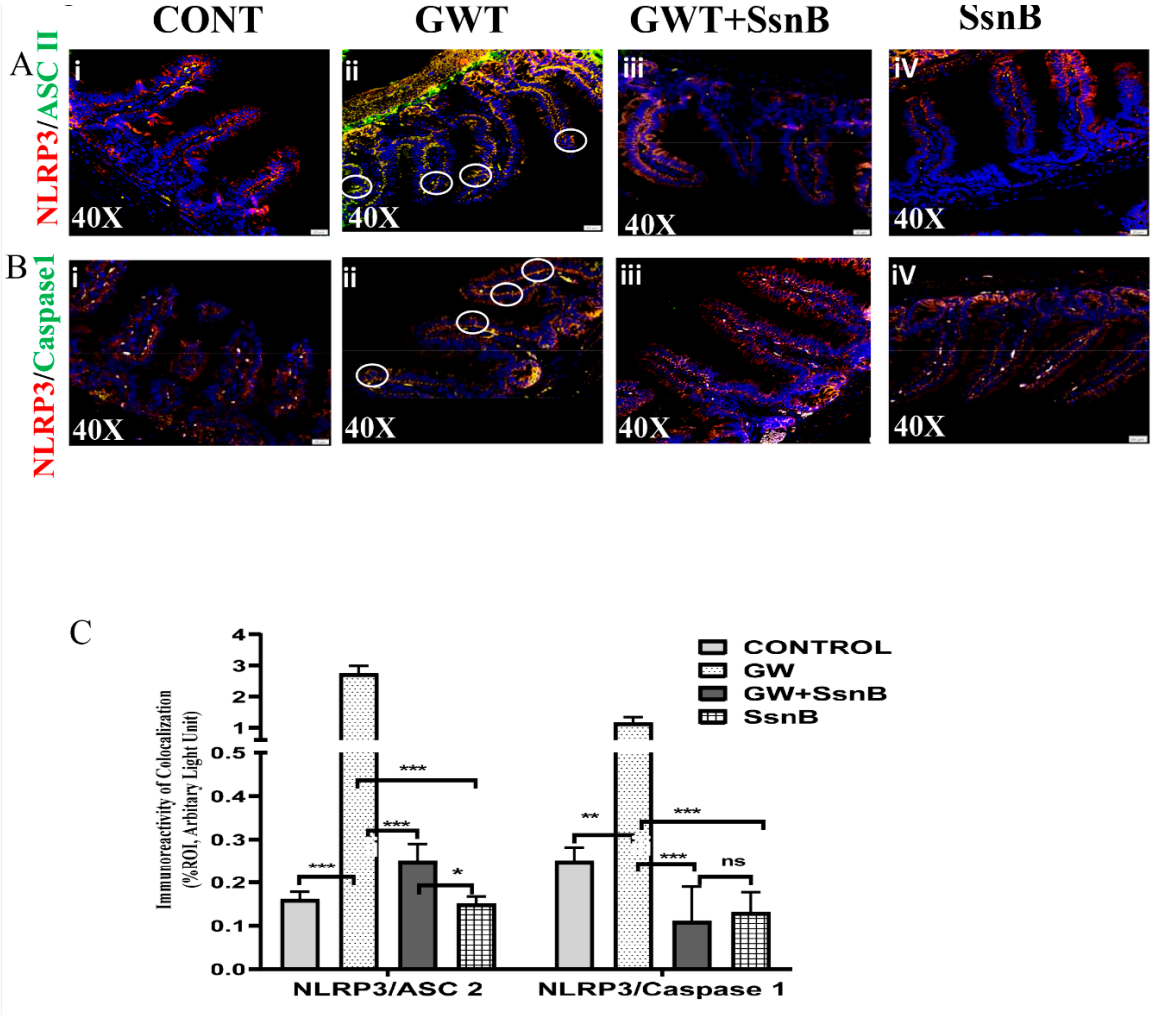

Figure 4. SsnB attenuates GW chemical-induced nod-like receptor protein 3 (NLRP3) inflammasome activation in the GWI mouse model. Activation of TLR4 signaling by HMGB1 may further activate the NLRP3 inflammasome complex, which exacerbates a pro-inflammatory response through secretion of inflammatory cytokines. The colocalization study of NLRP3 (red)/ASC2 (green) (Figure 4A) and NLRP3 (red)/Caspase 1 (green) (Figure 4B) was performed using an immunofluorescence microscopy in small intestine sections of CONT ($n = 3$) (wild-type mice which were treated with vehicle), GW ($n = 3$) (GW chemical exposed group), GW + SsnB ($n = 3$) (group exposed with GW chemicals and SsnB), and SsnB ($n = 3$) (group treated with SsnB only). Colocalizations were observed in the epithelial cells of the villi as yellow dots and are marked in the representative images by white circles. Images were taken at 40× magnification. Bar graphs depicting morphometric analysis of NLRP3/ASC2 and NLRP3/Caspase 1 (Figure 4C) colocalizations are represented as mean ± SD of the %ROI (mean value calculated from three separate fields of a section of the small intestine). Significance was analyzed by unpaired T-test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.5. SsnB Attenuates HMGB1 Induced NLRP3 Activation in Primary Mouse Intestinal Epithelial Cells

Previous studies have shown that DAMPs like HMGB1 induced NLRP3 inflammasome activation in the small intestine [26]. To confirm that HMGB1 induced NLRP3 activation, we performed an in vitro study using primary mouse intestinal epithelial cells and performed immunofluorescence microscopy by dual-labeling cells with NLRP3 (red) and ASC2 (green) antibody. Results showed that colocalization events were increased in cells treated with HMGB1 (observed as yellow dots around the nucleus of the

cells in the cytoplasm and marked with a white circle) compared to the control (CONT) group ($p < 0.01$) (Figure 5Ai,ii,C). Notably, the events of colocalization were markedly decreased in groups treated separately with two different concentrations of SsnB (10 and 100 µg/mL) and HMGB1 compared to cells treated with HMGB1 alone ($p < 0.01$) (Figure 5Aiii,iv,v,vi,C). We wanted to confirm the role of SsnB in decreasing the NLRP3 expression. We inactivated SsnB by acetylation and treated primary mouse intestinal epithelial cells with HMGB1 and the pseudo form or the inactivated SsnB (at 10 and 100 µg/mL) and dual labeled for NLRP3 (red) and ASC2 (green) antibodies. We found that cells treated with the inactivated SsnB along with HMGB1 had increased colocalization events of NLRP3/ASC2 comparable to the cells treated with HMGB1, only suggesting that SsnB was solely responsible for the inhibition ($p < 0.01$) (Figure 5Avii.viii,ix,x,C). We further confirmed our results by probing for IL-1β with cell lysates treated with HMGB1, HMGB1, and SsnB (at two different concentrations) and HMGB1 with an inactivated SsnB (Figure 5B,D) by Western blot.

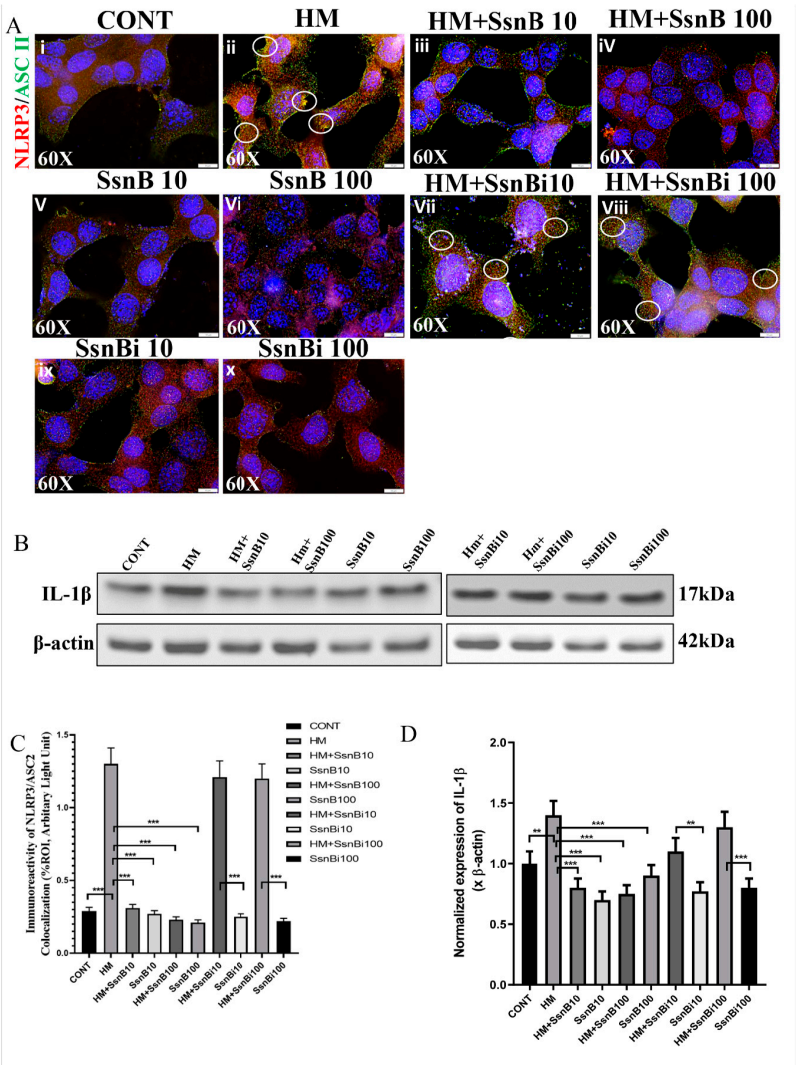

Figure 5. SsnB attenuates HMGB1 induced NLRP3 inflammasome activation in primary mouse intestinal epithelial cells. Mouse primary intestinal epithelial cells were treated with HMGB1 (HM), HMGB1 with SsnB at 10 µg/mL (HM + SsnB10) and 100 µg/mL (HM + SsnB100) concentrations and only SsnB at 10 µg/mL (SsnB10) and 100 µg/mL (SsnB100) concentrations. To confirm the role of SsnB in attenuating HMGB1 induced NLRP3 activation, the cells were further treated with inactivated or pseudo-SsnB along with HMGB1 at 10 µg/mL (HM + SsnBi10) and 100 µg/mL (HM + SsniB100) concentrations. Inactivated SsnB at the two concentrations (SsnBi10 and SsnBi100, respectively) were used as controls to compare with HM + SsnBi10 and HM + SsniB100. The colocalization study of NLRP3

(red)/ASC2 (green) (Figure 5A) was performed using immunofluorescence microscopy. Colocalizations were observed in the cytoplasmic region of the cells as yellow dots and are marked in the representative images by white circles. Images were taken at 60× magnification. Bar graphs depicting morphometric analysis of NLRP3/ASC2 (Figure 5C) colocalizations are represented as mean ± SD of the %ROI (mean value calculated from three separate fields of a sections). Significance was analyzed by unpaired T-test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Immunoblot analysis using mouse primary intestinal epithelial cell lysates was performed to further study the expression of IL-1β (Figure 5B). Bar graphs depicting densitometric analysis of immunoblots represented mean ± SD ($n = 3$), which were normalized with β-actin. Significance was analyzed by unpaired T-test where ** $p < 0.01$, *** $p < 0.001$ (Figure 5D).

3.6. SsnB Improves GW Chemical-Induced Neuroinflammation in Mouse GWI Model

Studies conducted by us and others have reported that GW chemical exposure led to neuroinflammation with increased expression of pro-inflammatory cytokines in a murine model of GWI [6,10]. We wanted to study whether SsnB induced improvement of gut health could consequently improve GW chemical-induced neuroinflammation. Immunohistochemistry was performed with HMGB1, IL-1β, and BDNF antibodies in mouse brain sections and results showed that expression of HMGB1 and IL-1β were significantly increased in the GW group (as observed by immunoreactivity in the frontal cortex region of the brain) compared to the control group ($p < 0.01$) (Figure 6Ai,ii,Bi,ii,D,E). Moreover, the expression of HMGB1 and IL-1β was significantly decreased in the GW + SsnB group and group treated with SsnB only ($p < 0.01$) (Figure 6Aiii,iv, Biii,iv,D, E). We also found that expression of BDNF, an important marker for neuronal plasticity [27] was markedly decreased in the GW chemical treated group compared to the control group ($p < 0.01$) (Figure 6Ci,ii,F). However, the expression of BDNF was found to be increased in GW + SsnB (not statistically significant) and in the group treated with SsnB only ($p < 0.05$) (Figure 6Ciii,iv,F).

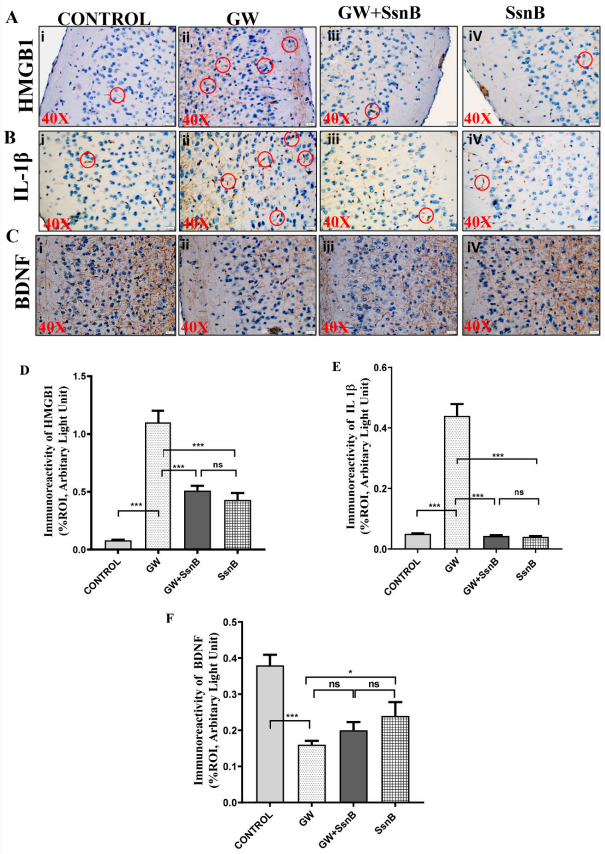

Figure 6. Administration of SsnB improves GW chemical-induced neuroinflammation in the mouse GWI model. Representative immunohistochemistry images of frontal cortex region showing immunoreactivity (marked with red circles) for HMGB1 (Figure 6A), IL-1β (Figure 6B), and neuronal plasticity marker BDNF (Figure 6C) in CONT (n = 3) (wild-type mice which were treated with vehicle), GW (n = 3) (GW chemical exposed group), GW + SsnB (n = 3) (group exposed with GW chemicals and SsnB), and SsnB (n = 3) (group treated with SsnB only). Images were taken in 40× magnification. Bar graphs depicting morphometric analysis of HMGB1 (Figure 6D), IL-1β (Figure 6E), and BDNF (Figure 6F) immunoreactivity are represented as mean ± SD of the %ROI (mean value calculated from three separate fields of a section of frontal cortex). Significance was analyzed by unpaired T-test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.7. SsnB attenuates GW Chemical-Induced TLR4 Activation and Inflammation Astrocytes of the GWI Mouse Model.

Astrocytes have been reported to play an essential role in the regulation of neuroinflammation in the brain. This occurs through the participation of TLR4 receptors that are shown to have a pivotal role in the activation of astrocytes [28]. In this study, we wanted to examine whether SsnB can downregulate GW chemical-induced expression of TLR4 in astrocytes. On dual-labeling mouse brain sections with GFAP (green) and TLR4 (red) antibodies, we found that TLR4/GFAP colocalization events (observed in the hippocampal region of the brain) were significantly decreased in the GW + SsnB group as compared with the GW group ($p < 0.01$) (Figure 7Aiii,iv,C). Notably, the highest colocalization events were observed in the GW group when compared to the control (*** $p < 0.001$) (Figure 7Ai,ii,C). In order to study astrocyte inflammation, we performed dual-labeling of GFAP (green) with S100B (red) antibodies. Results showed that GFAP/S100B colocalization events were significantly increased in the GW group compared to the control (*** $p < 0.001$) (Figure 7Bi,ii). In the GW + SsnB group, the events of colocalization were significantly decreased compared to the GW group (*** $p < 0.001$) (Figure 7Biii,iv,C). Further, we went on to study the mechanism through which SsnB attenuates inflammation in astrocytes. Western blot analysis for Myd88 and NFKB was performed using mouse brain lysates. Our results showed that the expression of Myd88 was decreased in the GW + SsnB group compared to the GW group, whereas the expression of phosphorylated NF-κB was significantly decreased in the GW + SsnB group as compared to the GW group ($p < 0.01$) (Figure 7D,E,F).

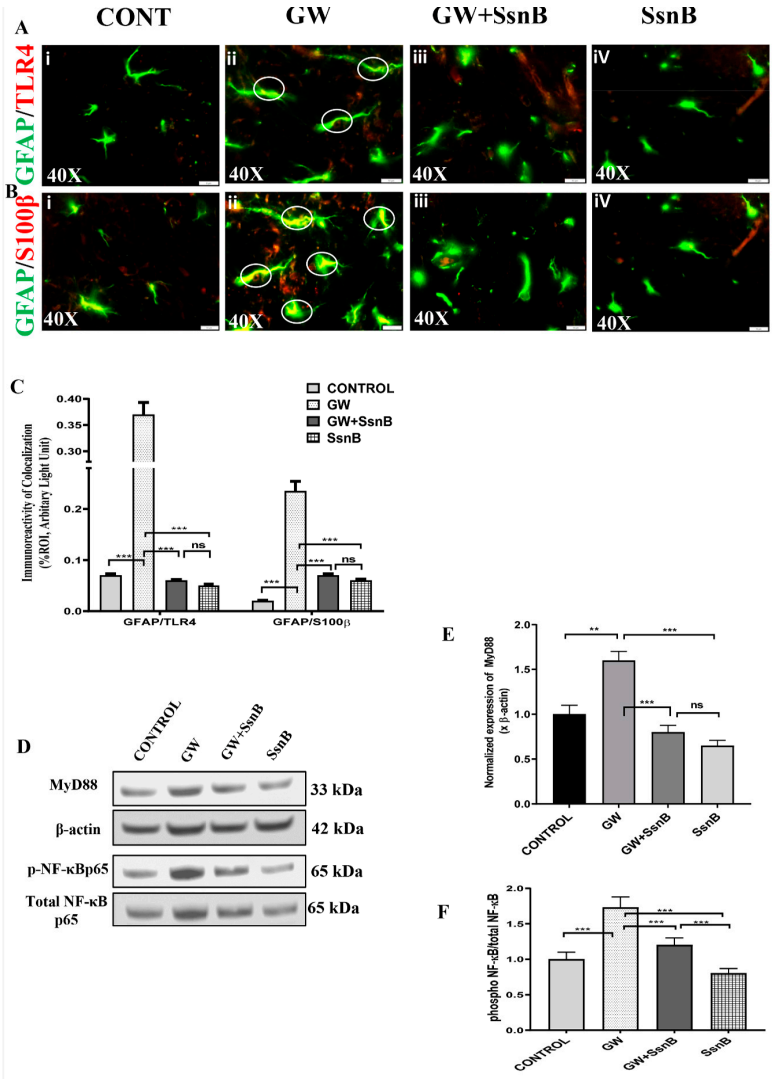

Figure 7. Treatment with SsnB attenuates GW chemical-induced HMGB1 mediated TLR4 activation and inflammation in the hippocampal region in the GWI mouse model. Increased serum expression of HMGB1 in the GW chemical treated mouse group suggests that HMGB1 may cause inflammation in ectopic sites. Colocalization study of TLR4 (red)/GFAP (green) (Figure 7A) and S100B (red)/GFAP (green) (Figure 7B) was performed using immunofluorescence microscopy in the hippocampal region in the brain of CONT (n = 3) (wild-type mice which were treated with vehicle), GW (n = 3) (GW chemical exposed group), GW + SsnB (n = 3) (group exposed with GW chemicals and SsnB), and SsnB (n = 3) (group treated with SsnB only). Colocalizations were observed in the astrocytes (labeled with GFAP) as yellow dots and are marked in the representative images by white circles. Images were taken at 40× magnification. Bar graphs depicting morphometric analysis of TLR4/GFAP and S100B/GFAP (Figure 7C) colocalizations are represented as mean ± SD of the %ROI (mean value calculated from three separate fields of a section of the small intestine). Significance was analyzed by unpaired T-test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Immunoblot analysis was performed using protein extracted from frontal cortex lysates to further study the role of SsnB in the expression of TLR4 signaling molecules MyD88, NF-κB (total p65 and phosphorylated p65) (Figure 7D). Bar graphs depicting densitometric analysis of MyD88 immunoblot normalized with β-actin represented mean ± SD (n = 3) (Figure 7E). Bar graphs depicting densitometric analysis of phosphorylated p65 normalized with total p65 (Figure 7F). Significance was analyzed by unpaired T-test where ** $p < 0.01$, *** $p < 0.001$.

3.8. SsnB Suppresses HMGB1 Induced Expression of GFAP/S100B in Mouse Primary Astrocytes

In this study, we have found that the concentration of serum HMGB1 was highest in the GW chemical treated group as compared to control and GW + SsnB groups as quantified by ELISA. This led us to hypothesize that the circulatory HMGB1 may cause inflammation in distant sites from the source, i.e., the small intestine. We treated mouse primary astrocytes with HMGB1 and SsnB (at 10 and 100 µg/mL concentration) and performed immunofluorescence microscopy by dual-labeling with GFAP (green) and S100B (red) antibodies. We found that cells treated with HMGB1 and SsnB had marked decreased by in colocalization events with GFAP/S100B as compared to cells treated with HMGB1 only ($p < 0.01$) (Figure 8i-vi,B). Cells treated with HMGB1 and inactivated SsnB (at 10 and 100 µg/mL concentration) gave results similar to cells treated with HMGB1 ($p < 0.05$) (Figure 8vii,viii,ix,x,B).

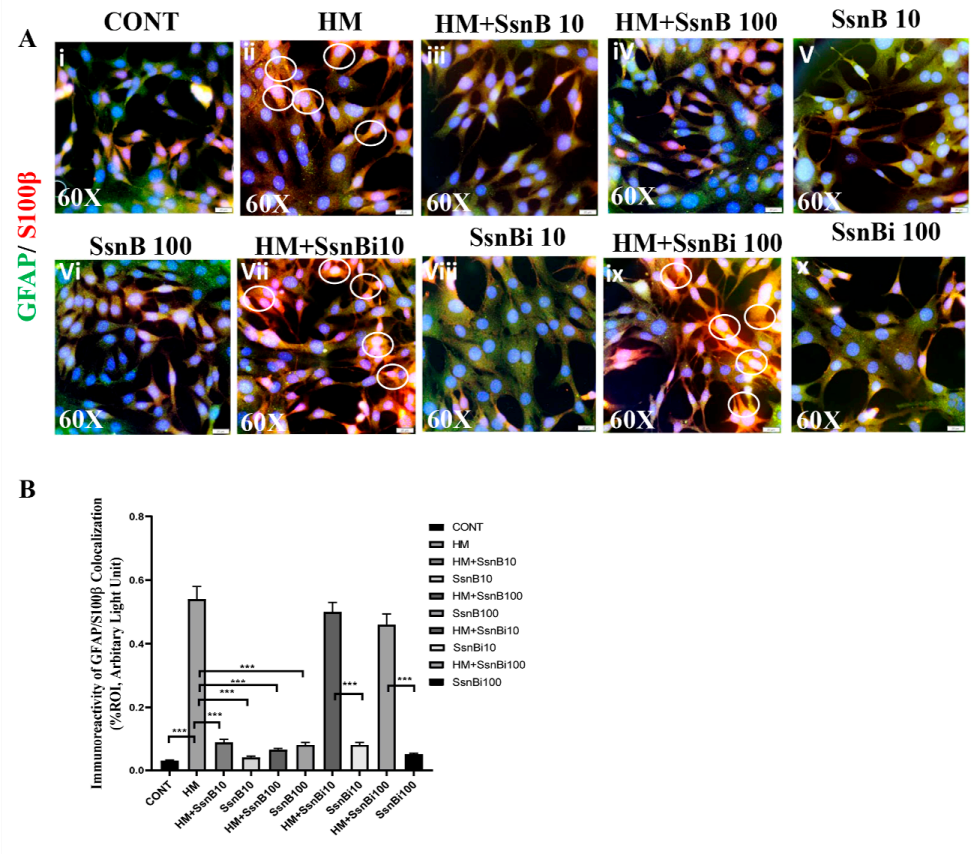

Figure 8. SsnB reduces HMGB1 induced expression of inflammatory markers GFAP/S100B in mouse primary astrocytes. Mouse primary astrocyte cells were treated with HMGB1(HM), HMGB1 with SsnB at 10 µg/mL (HM + SsnB10) and 100 µg/mL (HM + SsnB100) concentrations and only SsnB at 10 µg/mL (SsnB10) and 100 µg/mL (SsnB100) concentrations. The cells were also treated with inactivated or pseudo-SsnB along with HMGB1 at 10 µg/mL (HM + SsnBi10) and 100 µg/mL (HM + SsniB100) concentrations. Inactivated SsnB at the two concentrations (SsnBi10 and SsnBi100, respectively) were used as controls to compare with HM + SsnBi10 and HM + SsniB100. Immunofluorescence microscopy was used to study the inflammation induced by HMGB1 by dual-labeling the cells with S100B (red)/GFAP (green). Colocalizations were observed in the cytoplasmic region of the cells as yellow dots and are marked in the representative images by white circles (Figure 8A). Images were taken at 60× magnification. Bar graphs depicting morphometric analysis ofS100B/GFAP (Figure 8B) colocalizations are represented as mean ± SD of the %ROI (mean value calculated from three separate fields of a section). Significance was analyzed by unpaired T-test where ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

Our results showed that SsnB administration, a known TLR4 antagonist in mice that were pre-exposed to GW chemicals, presented a unique microbiome signature when compared to GW chemical-induced alteration of gut microflora. SsnB increased the relative abundance of butyrogenic bacteria known for their role in improving immune and gut health when compared to the GW chemical treated mouse group. The relative abundance of *Anaeroplasma*, a group of commensal bacteria that maintains the mucosal IgA level strengthening the gut barrier, was increased by three folds in the mouse group treated with SsnB and GW chemicals (GW + SsnB) compared to the GW chemical treated mouse group [29]. Dietary supplements that include nutraceuticals embedded in diets or consumed as a supplement have shown significant therapeutic benefits against chronicity of multisymptomatic gastrointestinal disease [30]. We and others have shown that dietary short-chain fatty acids or diet per se play a role in GWI pathogenesis [9,31]. The probiotic and pro-butyrogenic functions of SsnB that was observed in the mouse model was significant and a novel outcome. SsnB was introduced in the mouse model through the intraperitoneal route. Hence, it may be the inherent anti-inflammatory property of SsnB primarily by preventing TLR4 mediated signaling that decreases the GW chemical-induced inflammation in the gut leading to the restoration of gut dysbiosis and not its direct actions on the microflora per se. The increase in butyrogenic bacteria may also be due to a strong anti-inflammatory microenvironment in the gut epithelial cells that may differentially regulate colonization of bacterial species.

Interestingly, we found that the relative abundance of *Akkermansia* was decreased in the mouse group treated with SsnB and GW chemicals (GW + SsnB) compared to the GW chemical exposed group of mice. *Akkermansia* is a part of gut commensal microbiota that also plays an essential role in maintaining the metabolic and immunological functions along with maintaining the level of tight junction proteins in the healthy human gut [32]. It has been established that *Akkermansia* imparts its protective functions through TLR2 and TLR4 activation by its pili-like protein Muc-T and Amuc_1100, thereby secreting anti-inflammatory cytokine interleukin-10 (IL-10) [21]. In our study, blocking TLR4 activation by SsnB might have resulted in the decrease of *Akkermansia* population. However, the mechanism needs to be further investigated, and our study is limited to define the cause of its decrease. Our observation of a decrease in Genus OTU percent of *Akkermansia* is not reflective of depletion of that species, rather it only shows a comparative decrease vis a vis other groups. The decrease in *Akkermansia* species might also be explained by the above rationale of restrictive colonization of this species due to a strong anti-inflammatory effect of SsnB rather than the direct effect of the drug on the growth of the bacteria in the gut lumen though the above argument is speculative at this point. The above observation of a decrease in genus OTU percent for *Akkermansia* perhaps also highlights the importance of intact TLR4 signaling in the gut for launching a robust immune response. A complete blockage of TLR4 signaling in the gut by TLR4 antagonists such as SsnB might not augur well for the overall gut health though the anti-inflammatory effect of SsnB appears to attenuate the inflammation associated with GWI. An altered *Akkermansia* species abundance may subject the GWI-mouse for yet unknown gut-related health risks though its GWI-related inflammation may have been cured.

Results showed that the expression of tight junction proteins that were altered on GW chemical exposure resulting in a possible gut leaching observed in a similar study were restored to control levels following SsnB administration. Apparently, SsnBs role as an antagonist to TLR4 and its effect in decreasing Claudin 2 and increasing Occludin might have resulted from blockage of TLR4 downstream signaling in the epithelial cells of the gut. Claudin 2, a gut barrier protein is known to be upregulated in inflammation and has a pronounced role in causing gut barrier integrity loss [33]. Studies have shown that TLR4 signaling-induced pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-6, and IL-17 cause an increase in the levels of Claudin 2 via the phosphoinositide 3-kinase (PI3 Kinase) pathway [34]. A strong anti-inflammatory role of SsnB by blocking TLR4 signaling might have caused the observed decrease in Claudin 2 in our model. However, other studies show that TLR4 is an

important molecule in recognizing microbial motifs and may be important for maintaining gut barrier integrity as reviewed elsewhere by other mechanisms not related to the transcription of gut barrier proteins [35]. Our observed effects of SsnB may be primarily through TLR4 antagonism, but other cellular functions of this molecule cannot be ruled out.

We further went on to study the TLR4 activation pathway that plays a pivotal role in gastrointestinal inflammation in GWI. Studies show that HMGB1 released by the damaged intestinal epithelial cells may act as a ligand for TLR4 thereby activating a signaling cascade that further exacerbates gastrointestinal inflammation in IBD conditions [36,37]. Our results showed that GW chemical treated mice had an increased binding of HMGB1-TLR4, as seen in fluorescent microscopy that was decreased in mice groups that were exposed to GW chemicals and received SsnB. SsnB has already been established as a molecule that downregulates TLR4 activation by reducing the binding of MyD88 and decreasing NF-κB p65 activation [13]. In our results, we found that SsnB decreased TLR4-MyD88 binding and NF-κB p65 activation in mice groups administered with GW chemicals and SsnB compared to the GW chemical exposed mice group. Activation of TLR4 by HMGB1 may further activate cytosolic NLRP3 inflammasomes by recruiting adaptor protein ASC2 and Caspase 1 in intestinal epithelial cells that lay the groundwork for secretions of pro-inflammatory cytokines IL-1β and IL-18 [26]. Our study further reports that SsnB treatment with GW chemicals decreased TLR4 mediated NLRP3 activation through a decrease in NLRP3-ASC2 and NLRP3-Caspase1 complex formation in intestinal epithelial cells. NLRP3-inflammasomes are pivotal in disease-specific inflammatory pathways [38]. We recently reported their role in GWI and inflammation persistence (Kimono D et al., Neurosci Insights-In press). This was confirmed by an in vitro study by priming mouse primary intestinal cells with HMGB1 and treating with two different concentrations of SsnB (10 and 100 μg/mL) where NLRP3 activation was significantly decreased by SsnB. To further confirm the role of SsnB in the mechanistic pathways in vitro, we degraded the functional domain of SsnB by acetylation of the primary moiety and generating an inactivated or pseudo-SsnB. We found that the treatment of cells with inactivated SsnB with HMGB1 produced results similar to HMGB1-only treated cells. The above data confirmed SsnB to be the sole compound responsible for NLRP3 inactivation. However, the present study is limited in its more in-depth mechanistic approach about the structure of SsnB since it does not probe or characterize of the chemical moiety of acetylated SsnB. Notably, gastrointestinal inflammation is also triggered by activation of the enteric glial cells as shown by us previously [7]. Though we did not explore the role of SsnB in modulating the glial cells that are an integral part of the enteric nervous system, it is worth studying the TLR4 antagonism in these pathways [10,36].

Gulf War Illness is a neuroimmune disease [37]. Neuroinflammation and cognitive disorders induced likely by GW chemicals are observed to be the most commonly occurring symptoms of veterans with GWI [3,39]. We and others have found that neuroinflammation in GWI mouse models causes increased expression of pro-inflammatory cytokine such as IL-1β, IL-6, TNF-α, and DAMPs like HMGB1 in the brain, damaged blood-brain barrier (BBB) through alteration of tight junction protein, decreased expression of neuronal plasticity markers, and activation of TLR4 in the brain [6,10,40,41]. Results showed that SsnB exposure significantly decreased elevated expression of HMGB1 and IL-1β in GW groups. Expression of BDNF was also found to increase with the SsnB treatment though not significantly. We also found that SsnB reversed the GW chemical-induced inflammatory phenotype of brain astrocytes through a decrease in TLR4 activation and expression of inflammatory molecule S100B. An increased level of circulatory HMGB1 observed in the serum of GW mice may act as one of the mediators crossing the damaged blood-brain barrier and causing ectopic inflammation. Release of exosomes during gut dysbiosis containing HMGB1 may be the possible means for the transfer of the above DAMPs across BBB [42]. However, the present study could not decipher whether TLR4 activation occurrs by circulatory HMGB1 or endogenous HMGB1 secreted by neighboring damaged tissues such as activated microglia The interconnectivity of the astrocytes with other neurons is extensive, impacting significantly the neurovascular units of the brain [43]. It has been reported that the downregulation of NF-κB activation in astrocytes decreases inflammation and aids in recovery from

spinal cord injury [43]. The results in vitro of SsnB significantly decreasing HMGB1-TLR4 pathway activation via downregulation of NF-Kb phosphorylation is proof of its role in blocking the TLR4 signaling-induced inflammation.

The above experiments in deciphering the role of SsnB was carried out in an in vivo model of GWI pathology. The model is an adaptation from various other rodent models of GWI pathogenesis that mainly rely on GW-related chemical exposures and consumption of pharmaceuticals prescribed to the troops at that time. The prescribed drug used in our model is Pyridostigmine Bromide (PB). It is worth noting that not one model is a perfect platform for investigating GWI pathogenesis since exposures during that time period varied widely [5,36,40,41]. The use of an insecticide such as Permethrin is representative of the harmful effects of these classes of compounds [3]. It is important that advancing GWI pathogenesis research and testing preclinical efficacies should include multiple GWI models. Further exposure routes for insecticides such as Permethrin should also be tried intranasally to better model the effects in GWI.

5. Conclusions

The evidences presented in this study both in vivo and in vitro support a strong therapeutic role of SsnB, a relatively non-toxic nutraceutical in GW chemical-induced neuroinflammation and can be rapidly cleared for a clinical trial and approved for treatment in veterans with GWI.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3425/10/8/532/s1. Figure S1: Graphical representation of percentage relative abundance of microbiome at phylum level (bacteroidetes and firmicutes) that were significantly altered. The groups compared are CONT (n = 3) (wild-type mice which were treated with vehicle), GW (n = 3) (GW chemical exposed group), GW + SsnB (n = 3) (group exposed with GW chemicals and SsnB), and SsnB (n = 3) (group treated with SsnB only).

Author Contributions: Conceptualization, S.C.; data curation, S.C.; formal analysis, D.B. and A.M.; funding acquisition, S.C.; investigation, D.B., A.M., P.S., D.K., S.S., R.K.S., and S.C.; methodology, D.B., A.M., P.S., and R.K.S.; resources, S.C.; software, D.B. and R.K.S.; supervision, S.C.; writing—original draft, D.B. and S.C.; writing—review and editing, R.K.S., P.J., K.S., R.H., N.K., M.N., P.N., and S.C. All authors have read and agreed to the published version of the manuscript.

Funding: Funding for this project was provided by the DoD-IIRFA grant W81XWH1810374, VA merit award I01 CX001923-01 and National Institutes of Health Awards 2-P20-GM-103641-06, and P01-AT-003961 (Project 4) to S. Chatterjee; and P01-AT-003961, P20-GM-103641, to M. Nagarkatti and P. Nagarkatti "This material is based upon work supported (or supported in part) by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development vide grant no. CX001923-01" to Saurabh Chatterjee. Chatterjee is a 5/8th employee with the US Department of Veterans Affairs.

Acknowledgments: We are grateful to the Instrument Resource Facility (University of South Carolina School of Medicine) and AML Labs (Baltimore MD) for providing excellent technical services. The authors thank Ms. Yin Zhong for generating the microbiome data.

Conflicts of Interest: The authors declare no conflict of interest.

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Brain, Behavior, and Immunity 87 (2020) 498–507

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

In-vivo imaging of neuroinflammation in veterans with Gulf War illness

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A B S T R A C T

Gulf War Illness (GWI) is a chronic disorder affecting approximately 30% of the veterans who served in the 1991 Gulf War. It is characterised by a constellation of symptoms including musculoskeletal pain, cognitive problems and fatigue. The cause of GWI is not definitively known but exposure to neurotoxicants, the prophylactic use of pyridostigmine bromide (PB) pills, and/or stressors during deployment have all been suspected to play some pathogenic role. Recent animal models of GWI have suggested that neuroinflammatory mechanisms may be implicated, including a dysregulated activation of microglia and astrocytes. However, neuroinflammation has not previously been directly observed in veterans with GWI. To measure GWI-related neuroinflammation in GW veterans, we conducted a Positron Emission Tomography (PET) study using [^{11}C]PBR28, which binds to the 18 kDa translocator protein (TSPO), a protein upregulated in activated microglia/macrophages and astrocytes.

Veterans with GWI (n = 15) and healthy controls (HC, n = 33, including a subgroup of healthy GW veterans, HC_{VET}, n = 8), were examined using integrated [^{11}C]PBR28 PET/MRI. Standardized uptake values normalized by occipital cortex signal (SUVR) were compared across groups and against clinical variables and circulating inflammatory cytokines (TNF-α, IL-6 and IL-1β). SUVR were validated against volume of distribution ratio (n = 13).

Whether compared to the whole HC group, or only the HC_{VET} subgroup, veterans with GWI demonstrated widespread cortical elevations in [^{11}C]PBR28 PET signal, in areas including precuneus, prefrontal, primary motor and somatosensory cortices. There were no significant group differences in the plasma levels of the inflammatory cytokines evaluated. There were also no significant correlations between [^{11}C]PBR28 PET signal and clinical variables or circulating inflammatory cytokines.

Our study provides the first direct evidence of brain upregulation of the neuroinflammatory marker TSPO in veterans with GWI and supports the exploration of neuroinflammation as a therapeutic target for this disorder.

1. Introduction

Gulf War Illness (GWI) is a chronic disorder affecting approximately 30% of the nearly 700,000 veterans who served in the 1991 Gulf War (Binns et al., 2008). It is characterised by a constellation of symptoms including musculoskeletal pain, fatigue, and cognitive/affective decrements. GWI has been suspected to be caused by exposure to neurotoxicants (including the nerve gas sarin and/or pesticides used to prevent insect-borne diseases), the prophylactic use of pyridostigmine bromide (PB) pills (an acetylcholinesterase inhibitor commonly used to protect troops from the harmful effects of nerve agents), and/or the experience of physical stressors (e.g., extreme temperature changes, sleep deprivation, physical exertion) during deployment (Binns et al., 2008; Fukuda et al., 1998; Janulewicz et al., 2018; Maule et al., 2018; Steele et al., 2012; Sullivan et al., 2018).

The wide range of symptoms of GWI is indicative of a complex underlying pathophysiology, for which the etiology has remained largely undetermined. Many of the symptoms reported by veterans with GWI are indicative of central nervous system (CNS) dysfunction, and indeed this has been corroborated by structural and functional neuroimaging and biomarker studies (Abou-Donia et al., 2017; White et al., 2016). Dysfunction of the CNS includes alterations in brain white

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https://doi.org/10.1016/j.bbi.2020.01.020
Received 17 October 2019; Received in revised form 27 January 2020; Accepted 30 January 2020
Available online 04 February 2020

matter (e.g., reduced volume and increased mean diffusivity; [Heaton et al., 2007; Rayhan et al., 2013b]), decreases in metabolite levels (e.g., lower NAA/creatine ratio; [Menon et al., 2004]), decreases in cerebral blood flow (Haley et al., 2009), reduced gray matter volume (Chao et al., 2010; Rayhan et al., 2013a) and altered gray matter activity in response to behavioral, sensory and chemical stimuli (Calley et al., 2010; Gopinath et al., 2012; Haley et al., 2009). There are also reports and reviews documenting neurobehavioral dysfunction such as slower motor function, poorer visual and verbal memory and worse attention in GWI, and studies have shown that these symptoms are associated with inflammatory cytokines and reduced hippocampal volume (Janulewicz et al., 2018; Jeffrey et al., 2019; O'Donovan et al., 2015; Sullivan et al., 2018).

While the exact pathophysiology of GWI remains unknown, recent studies suggest a possible role for neuroinflammation, and dysregulated activation of microglia and astrocytes (Madhu et al., 2019b; Parihar et al., 2013). Microglia are the resident macrophages of the CNS and rapidly activate in response to pathological danger signals (Kreutzberg, 1996). Acutely, this response is essential for survival, as it allows for the identification of a potentially harmful event, limiting its impact and favoring its resolution. However, overactivation of microglia can lead to production of excessive pro-inflammatory cytokines and excitotoxins, which can be deleterious (Mika, 2008). Similarly, astrocytes can enact responses to pathological events that are adaptive in the acute phase, but can sometimes become dysregulated and pathogenic (Pekny and Pekna, 2014; Pekny et al., 2014). It has been proposed that such aberrant activation can be the result of glial cells being 'primed' by prior pathological events including systemic infections, toxic environmental exposures, and trauma, making them more vulnerable to subsequent stressors, in a 'two-hit' model (Blaylock and Maroon, 2011; Perry et al., 1985; Watkins et al., 2007a). In GWI, multiple neurotoxicant chemical exposures (pesticides, PB, sarin), compounded with the experience of mental or physical stressors, has been suggested to be among potential triggering mechanisms for the chronic symptoms and neuroinflammation in GWI (Binns et al., 2008). For instance, in a recent mouse model investigation, the exposure to a sarin surrogate (DFP) induced widespread neuroinflammation in multiple brain areas such as the frontal cortex, hippocampus, cerebellum and the hypothalamus, and these effects were exacerbated by pre-exposure to corticosterone, the endogenous glucocorticoid typically released in conditions of high physiological stress (O'Callaghan et al., 2015). Despite these animal observations, to our knowledge, GWI-related neuroinflammation has never been demonstrated in humans.

Here we hypothesized that veterans with GWI would demonstrate neuroinflammation in the CNS compared to veterans without GWI and healthy civilians. More specifically, we hypothesized that the pattern of neuroinflammation would be similar to that observed in participants with fibromyalgia (Albrecht et al., 2019). Both fibromyalgia and GWI are in fact accompanied by chronic sickness behavior, with similar hallmarks such as widespread musculoskeletal pain, fatigue, and cognitive difficulties (Arnett and Clark, 2012; Maule et al., 2018; O'Callaghan and Miller, 2019; Wolfe et al., 1990), suggesting the possibility that these conditions present shared mechanisms.

In this study, we used positron emission tomography (PET) and the radioligand [^{11}C]PBR28 to evaluate and document the role of neuroinflammation in veterans with GWI. [^{11}C]PBR28 binds to the 18-kDa translocator protein (TSPO) (Briard et al., 2008; Brown et al., 2007), a mitochondrial protein that is expressed at very low levels in the healthy CNS but becomes dramatically upregulated by activated microglia/macrophages and reactive astrocytes, and is therefore considered a surrogate marker of neuroinflammation (Cagnin et al., 2007; Lacor et al., 1996; Lavisse et al., 2012; Rupprecht et al., 2010).

2. Materials and methods

2.1. Study design

The study was conducted at the Athinoula A. Martinos Center for Biomedical Imaging at Massachusetts General Hospital. The institutional review board and the Radioactive Drug Research Committee approved this study. All participants gave written informed consent.

2.2. Participants

Fifteen veterans with GWI (12 males, 51.1 ± 1.3 years old [mean ± SD]), and 33 healthy controls (17 males, 47.9 ± 12.6 years old; HC) were recruited for the study. The HC sample included 8 healthy veterans of the Gulf War (7 males, 51.4 ± 2.1 years old; HC_{VET}) and 25 healthy civilians (10 males, 46.8 ± 2.8 years old; HC_{CIV}). All GWI veterans met the Kansas diagnostic criteria for GWI which requires endorsing at least three out of six symptom domains (fatigue, pain, neurological, skin, gastrointestinal, and respiratory) and either moderate or multiple mild symptoms within each domain (Steele, 2000). Veterans not meeting Kansas GWI or other exclusionary criteria were considered healthy controls. Participants from all groups were excluded for the presence of a history of major psychiatric illness, neurological illness, cardiovascular disease, inability to communicate in English and for contraindication for PET/MR scanning (e.g., pacemaker, metallic implants, pregnancy, etc.).

2.3. Behavioral visit

All participants in the study were asked to complete the Beck Depression Inventory (BDI; [Beck et al., 1961]) and the Brief Pain Inventory (BPI; [Daut et al., 1983]). In addition, all veterans were asked to complete the 2011 American College of Rheumatology (ACR) self-report survey for the assessment of fibromyalgia symptoms (Wolfe et al., 2011), the Kansas GWI Questionnaire to detect and score the severity of GWI (Steele, 2000) and Conner's Continuous Performance Test III (CPT3) for the assessment of attention (Conners et al., 2000). During the visit, venous blood was drawn from all participants in order to have all participants genotyped for the Ala147Thr *TSPO* polymorphism, which predicts binding affinity to the radioligand (Owen et al., 2010; Owen et al., 2012). Subjects exhibiting the Thr/Thr genotype, which predicts low affinity binding status, were excluded from the imaging procedures, whereas participants with the Ala/Ala or Ala/Thr polymorphisms, which are associated with high and mixed affinity binding, respectively, were allowed to proceed.

2.4. Imaging visit

At the beginning of the imaging visit, a subset of subjects (n = 32) had venous blood collected to measure the level of circulating inflammatory cytokines, using the Meso Scale Discovery V-Plex Plus Proinflammatory Panel. Cytokine analyses were performed through a third-party vendor (Beantown Biotech, Natick MA). In this study we focused on IL-6, TNF-α and IL-1β because their level is commonly reported as altered in animal models and/or humans with pain disorders (Ji et al., 2013; Loggia et al., 2015) and/or GWI (Johnson et al., 2016; O'Donovan et al., 2015). Brain imaging was then performed with a Siemens PET/MRI scanner, consisting of a dedicated brain avalanche photodiode-based PET scanner in the bore of a Siemens 3T Tim Trio MRI (Kolb et al., 2012). Up to 15 millcurie (mCi) of [^{11}C]PBR28, produced in-house using a procedure modified from the literature (Imaizumi et al., 2007), were injected as an intravenous bolus, and dynamic PET were acquired for 90 min as described previously (Albrecht et al., 2018; Loggia et al., 2015). Because the HC_{CIV} were recruited through a different study protocol, they happened to have a significantly lower injected dose compared to GWI (GWI:

14.307 ± 1.07 mCi [529.4 ± 39.5 Mbq]; HC_{VET}: 14.307 ± 0.98 mCi [529.4 ± 36.2 Mbq]; HC_{CIV}: 12.262 ± 1.42 mCi [453.7 ± 56.1 Mbq; mean ± SD]; GWI vs HC_{CIV}: p = 0.002). However, there was no difference in dose between GWI and HC_{VET} (p = 0.99). For anatomical localization, spatial normalization and generation of attenuation correction maps (Izquierdo-Garcia et al., 2014b), a multi-echo MPRAGE (T1-weighted structural MRI) volume was also acquired (TR/TE1/TE2/TE3/TE4 = 2530/1.64/3.5/5.36/7.22 ms, flip angle = 7°, voxel size = 1 mm isotropic).

In 17 participants (6 veterans), a radial artery catheter was inserted and blood samples were collected at 6–10 s intervals for the first three minutes, followed by samples collected at 5, 10, 20, 30, 50, 70 and 90 min post [^{11}C]PBR28 injection. These data were collected for the purpose of creating a radiometabolite-corrected arterial input function to perform full kinetic modelling, in order to validate the semi-quantitative ratio metric used in the study (see below). Technical issues were encountered for 4 participants, thus the blood data from these participants were excluded from further analyses. Arterial blood processing was performed as previously described by Albrecht et al. (2018).

2.5. Imaging data preprocessing

From the [^{11}C]PBR28 PET data, standard uptake volume ratio (SUVR) images, from 60 to 90 min post-injection data, were generated as described previously (Albrecht et al., 2018; Loggia et al., 2015; Zurcher et al., 2015). Essentially, standard uptake volume (SUV) images, computed by normalizing radioactivity by injected dose/body weight, were attenuation corrected using a published MR-based method (Izquierdo-Garcia et al., 2014a). These SUV maps were then nonlinearly transformed to MNI space, smoothed with an 8 mm full width half-maximum Gaussian kernel, and then intensity-normalised by dividing them by the mean SUV extracted from the occipital cortex (identified using a label from the AAL atlas available in PMOD [Tzourio-Mazoyer et al., 2002]) to obtain SUVR maps. We have previously utilized this approach for quantification of [^{11}C]PBR28 PET data in patients with chronic low back pain, amyotrophic lateral sclerosis (Albrecht et al., 2018) and, more relevant for the current study, fibromyalgia (Albrecht et al., 2019), a condition with a clinical presentation similar to that of GWI. The lack of significant group differences in [^{11}C]PBR28 SUV signal in the occipital cortex was confirmed in this study when the GWI veterans were compared to all HCs (p = 0.81) and when they were compared with the subset of HC_{VET} (p = 0.87). In order to further support the validity of SUVR as an outcome metric, we compared SUVR against distribution volume (V_T) ratio (DVR), in a subset of participants for whom arterial plasma data were available (n = 13; detailed methods described in [Albrecht et al., 2018]). To this end, V_T was computed from “target regions” (ie. regions identified as statistically significant across groups in the voxel-wise analyses in this study; see below) as well as the occipital cortex, using radiometabolite-corrected arterial input function (AIF) and traditional 2-tissue-compartmental modelling. Each target region was divided by occipital cortex V_T to obtain DVR. In all evaluated regions, SUVR were strongly correlated with DVR ($8.7 \times 10^{-7} \leq p \leq 8.9 \times 10^{-3}$, $0.69 \leq r \leq 0.95$; Supplementary Fig. 1). These results provide support for the use of SUVR as a viable PET metric in our study.

2.6. Statistical analysis

Group differences were assessed with Student’s *t*-tests for continuous variables (age, clinical and cytokine variables) and chi-square tests for categorical variables (sex and genotype), using Statistica (TIBCO Software Inc., v.13). The main group analyses compared the GWI group with the whole HC group, taking advantage of the relatively large sample of controls. While differences in age between these groups did not meet our threshold for statistical significance (p = 0.34), the differences in sex distribution approached significance (p = 0.061). For this reason, comparisons between these groups included sex as a covariate. In addition, because *TSPO* genotype affects binding affinity (Owen et al., 2010; Owen et al., 2012), all PET analyses were also corrected for genotype. In addition to these main analyses, we compared the GWI group against the subset of healthy controls who were GW veterans (n = 8). These secondary, exploratory analyses were performed to evaluate whether the effects observed in the main analyses could be observed when contrasting groups that were better demographically matched and had comparable GW combat exposure. Because neither sex (p = 0.65) nor age (p = 0.67) were significantly different across these groups, analyses between GWI and HC_{VET} were only corrected for genotype.

Group analyses were performed using two strategies. First, because one of the hypotheses of this study was that GWI patients would demonstrate similar neuroinflammatory patterns as those observed in fibromyalgia patients, we performed ROI analyses using - as our a priori ROIs - statistically significant clusters from our previous study demonstrating increased [^{11}C]PBR28 signal in that patient group: primary motor/somatosensory cortex (M1/S1), dorsolateral prefrontal cortex (dlPFC), precuneus and anterior mid cingulate cortex (aMCC) (Albrecht et al., 2019). Next, a whole brain voxel-wise analysis was performed, in order to evaluate the possible presence of group differences in the [^{11}C]PBR28 signal beyond the boundaries of the a priori ROIs, as well as to localize any effects observed in the ROI analyses with higher spatial accuracy. Because the injected dose was significantly different between GWI and HC, these analyses were repeated including injected dose as a covariate in the analyses. These analyses were performed with FSL’s FEAT GLM tool (www.fmrib.ox.ac.uk/fsl, version 5.0.10). For ease of visualization of the cortical effects and for better comparison with the results of the fibromyalgia study, imaging results are visualized on a surface (FreeSurfer’s fsaverage) in the main manuscript. In addition, results were also overlaid onto MNI volumetric standard brain for visualisation of white matter and subcortical structures (Supplementary Fig. 3).

For visualization purposes, as well as for correlation analyses (see below), data were extracted from the significant clusters identified in the voxel-wise analyses comparing GWI and HC and anatomically split using labels from the Harvard-Oxford Cortical Structural Atlas (Centre for Morphometric Analyses, http://www.cma.mgh.harvard.edu/fsl_atlas.html).

In GWI patients, the [^{11}C]PBR28 signal from these ROIs was correlated with clinical variables (Kansas GWI score, fibromyalgia score, score on the fatigue item of the ACR self-report survey for the assessment of fibromyalgia symptoms, CPT3 hit reaction time, BPI pain and BDI), in order to evaluate potential association between neuroinflammation and GWI symptom severity, as well as with levels of circulating cytokines (IL-6, TNF-α and IL-1β), to explore the relationship between central and peripheral inflammation (correcting for genotype). Because these analyses were exploratory, the results in this case were not corrected for multiple comparisons.

3. Results

3.1. Participant characteristics

Demographic and other key characteristics for all participants are displayed in Table 1. As briefly mentioned in the methods section, there was no significant difference in sex or age between the GWI and HC groups, (p = 0.061 and 0.339, respectively). Differences in genotype distributions between these groups approached but did not reach statistical significance (p = 0.051). There was also no significant difference between GWI and HC_{VET} groups in sex, genotype, or age (p = 0.651, 0.435 and 0.919, respectively).

Table 1
Participant characteristics.

Participant characteristics			
	GWI	HC	GWI vs HC
N	15	33 (all) 8 (veterans)	–
Sex	12M; 3F	17M; 15F (all) 7M; 1F (veterans)	p = 0.06 p = 0.65
Age (years: mean ± sd)	51.1 ± 5.0	47.9 ± 12.6 (all) 51.4 ± 6.1 (veterans)	p = 0.34 p = 0.92
TSPO polymorphism	5H; 10M	21H; 12M (all) 4H; 4M (veterans)	p = 0.05 p = 0.44

3.2. Behavioral measures and blood cytokine levels

There was a significant difference between groups in all behavioral measures, with the GWI participants demonstrating higher Kansas GWI and fibromyalgia scores, fatigue, pain and depression except for the Conner's CPT3 HRT test (Table 2). There was no significant difference in levels of IL-6, IL-1β and TNF-α in veterans with GWI when compared to HC (p's $>$ 0.05; Table 2).

3.3. Imaging results: A priori ROI analyses

Compared to the HC group, GWI participants demonstrated significantly elevated [^{11}C]PBR28 PET signal in the dlPFC, precuneus and aMCC, i.e., three out of four of the a priori ROIs. The PET signal elevations in the dlPFC and precuneus remained statistically significant when GWI were compared with HC_{VET}, and additional signal elevations were observed in M1-S1, (Fig. 1).

3.4. Imaging results: voxel-wise group differences

The voxel-wise comparison between GWI and HC revealed widespread cortical [^{11}C]PBR28 PET signal elevations (and no regions of PET signal reduction) in GWI. These were observed both within the regions used as a priori ROIs in the analysis previously described (S1, M1, dlPFC, aMCC and precuneus) as well as additional regions (dorsomedial prefrontal cortex [dmPFC], paracingulate cortex, anterior cingulate cortex [ACC], ventral medial PFC [vmPFC] and posterior cingulate cortex [PCC]; Fig. 2A). Several of these regions [S1, M1, dlPFC, dmPFC, precuneus and the superior parietal lobule (SPL; Fig. 2B)] survived statistical significance when GWI were compared with the HC_{VET} subgroup. For display purposes, the mean SUVR values from a subset of regions are displayed in Fig. 2C. In addition, as injected dose was significantly different between groups when veterans with GWI were compared with HC, we ran an exploratory voxel-wise analysis, including injected dose as a covariate. This analysis yielded similar results (Supplementary Figure 2).

3.5. Imaging results: Regression analyses

Regions that showed elevations in SUVR in GWI compared to HC were selected as an ROI for correlations with clinical variables and circulating cytokine levels in the GWI group only, in exploratory analyses. In no region was there a significant correlation between SUVR signal in GWI and clinical variables or circulating inflammatory cytokines (Table 3).

4. Discussion

The current study provides in-vivo evidence of neuroinflammation in veterans with GWI. When compared to GW veterans without GWI and healthy civilians, veterans with GWI demonstrated elevated TSPO binding, as measured with [^{11}C]PBR28 PET. This marker of neuroinflammation demonstrated elevated levels throughout cortical areas such as precuneus, prefrontal cortex, and primary motor and somatosensory areas, as well as underlying white matter and the putamen. The neuroinflammatory signal elevations observed in GWI demonstrated spatial similarities to that observed in fibromyalgia (Albrecht et al., 2019), as we had hypothesized given the overlap in the clinical presentation of the two conditions. Fatigue, musculoskeletal pain, disturbed sleep, memory and attention deficits are some of the symptoms that affect both conditions (Binns et al., 2008; Clauw, 2014). In fact, GWI veterans are often diagnosed with fibromyalgia (Blanchard et al., 2019).

While this study represents the first report of in-vivo neuroinflammation imaging in veterans with GWI, these results conform to a body of preclinical research that has shown neuroinflammation in animal models of GWI (White et al., 2016). Such models have shown that the exposure to neurotoxicant chemicals such as irreversible acetylcholinesterase inhibitor (AChEi), organophosphate pesticides, nerve agents and prophylactic treatment with pyridostigmine bromide pills (PB; a reversible AChEi), i.e., the same compounds the veterans had been exposed to during the GW, induces chronic neuroinflammation (Banks and Lein, 2012; Koo et al., 2018; O'Callaghan et al., 2015; Ojo et al., 2014; Parihar et al., 2013). Additionally, pyrethroid pesticides, which were also widely used during the GW, mediate their action by opening voltage gated sodium channels which results in excessive neuronal firing (Hue and Mony, 1987), possibly inducing neurogenic inflammation both centrally (Xanthos and Sandkühler, 2013), as well as

Table 2
Participant clinical and cytokine variables.

Group clinical and cytokine variables			
	GWI	HC	GWI vs HC
Kansas GWI score	45.2 ± 18.86	1.75 ± 2.96 (veterans)	$p = 5.49 \times 10^{-6}$
ACR Fibromyalgia score	18.4 ± 6.98	1.25 ± 2.12 (veterans)	$p = 2.12 \times 10^{-6}$
ACR Fatigue score	2.1 ± 0.80	0 (veterans)	$p = 9.69 \times 10^{-7}$
Conner's CPT3 Hit Reaction Time	54.4 ± 8.81	47.88 ± 4.88 (veterans)	p = 0.103
BPI Pain Intensity	5.6 ± 1.42	0.27 ± 0.58 (all) 0.56 ± 0.95 (veterans)	$p < 0.0001$ $p = 2.57 \times 10^{-8}$
BDI	17.7 ± 9.92	1.62 ± 2.45 (all) 2.69 ± 3.65 (veterans)	$p = 1.12 \times 10^{-9}$ $p = 0.0008$
IL-6	3.28 ± 7.14	0.57 ± 0.57 (all) 0.87 ± 0.52 (veterans)	p = 0.128 p = 0.357
TNF-α	6.80 ± 6.37	3.63 ± 2.58 (all) 5.76 ± 2.15 (veterans)	p = 0.071 p = 0.664
IL-1β	2.61 ± 6.69	0.79 ± 0.66 (all) 0.65 ± 0.38 (veterans)	p = 0.359 p = 0.455

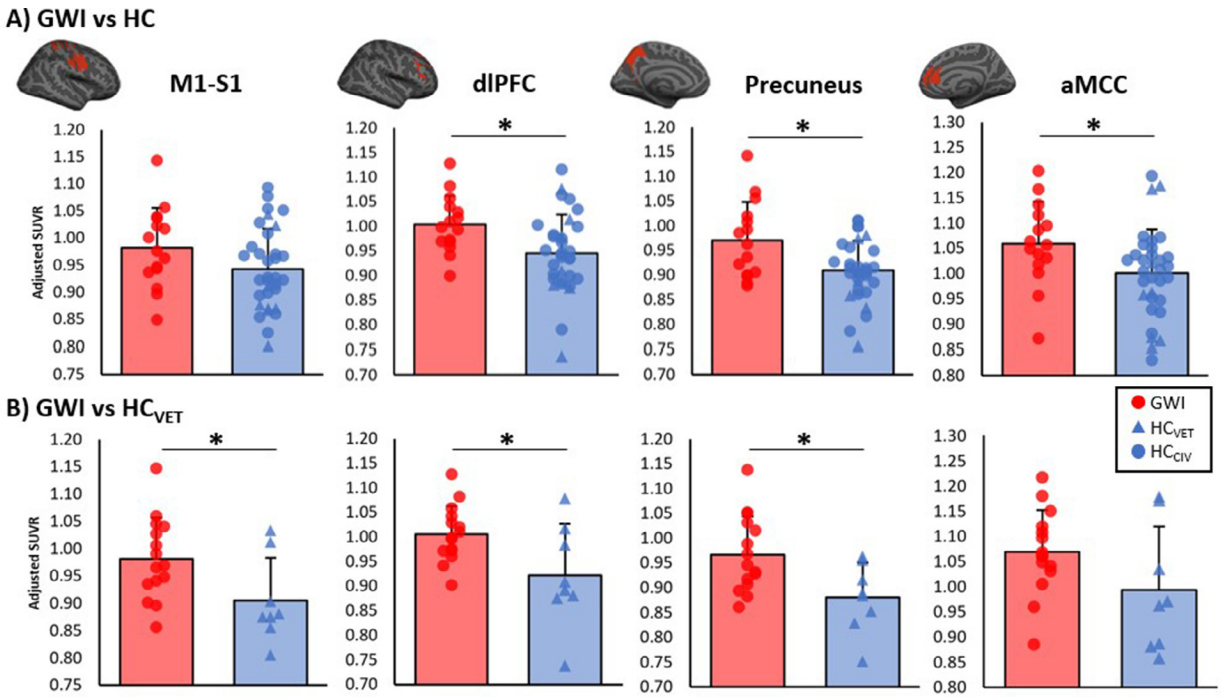

Fig. 1. ROI analyses. Group differences in [^{11}C]PBR28 standardized volume uptake (SUVR) in a priori ROIs. These regions were selected as they demonstrated [^{11}C]PBR28 PET signal elevations in fibromyalgia patients. Top panel: Average ± standard deviation SUVR extracted showing differences between GWI and HC (adjusted for genotype and sex). Bottom panel: Average ± standard deviation SUVR extracted showing differences between GWI and HC$_{VET}$ (adjusted for genotype). Surface projections of regions are displayed in red above the plots. *significant difference between groups ($p < 0.05$). M1 = primary motor cortex; S1 = primary somatosensory cortex; dlPFC = dorsolateral prefrontal cortex; aMCC = anterior mid cingulate cortex. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

peripherally (Chiu et al., 2012; Roosterman et al., 2006). While our study showed that veterans with GWI demonstrated elevations of central (TSPO PET signal) markers of inflammation, we found no evidence of elevations in peripheral (pro inflammatory cytokines) markers of inflammation.

In addition to neurotoxicant exposure, physical and mental stressors can induce neuroinflammation, perhaps compounding its effects. Indeed, some animal models of GWI are produced through the combination of exposure to GW-relevant neurotoxicant AChEi chemicals (sarin, pesticides, PB) and stress (White et al., 2016). In these models of GWI, astrogliosis and/or microglial activation have been reported within the prefrontal cortex, the hippocampus, striatum, hypothalamus, olfactory bulb and the cerebellum (O'Callaghan et al., 2015; Ojo et al., 2014; Parihar et al., 2013). Interestingly, O'Callaghan et al. (2015) found that these neuroinflammatory responses were greatly exacerbated when animals were pre-treated with rodent stress hormone corticosterone (CORT), administered at levels compatible with those observed with high physiological stress (O'Callaghan et al., 2015), possibly at the level that the veterans might have experienced given the harsh conditions that were prevalent during the Gulf War [e.g., extreme heat or cold, or sleep deprivation (Gifford et al., 2006)]. Indeed, studies have shown that sleep disturbances and heat stress can induce neuroinflammation (Chauhan et al., 2017; Zhu et al., 2012). Furthermore, these stressors, along with other brain insults including a history of mild traumatic brain injury (mTBI) can 'prime' glial cells for aberrant activation which might lead to chronic neuroinflammation (Blaylock and Maroon, 2011; Burda et al., 2016; Chen et al., 2014; Kuhlmann and Guilarte, 2000; Perry et al., 1985; Watkins et al., 2007a).

In this investigation, we have used [^{11}C]PBR28 to image TSPO binding as a marker of neuroinflammation. Though TSPO is constitutively expressed by many cell types, within the CNS, this protein is upregulated primarily or exclusively in glial cells during neuroinflammatory responses, and hence can be used as a sensitive marker of glial activation (Wei et al., 2013). Indeed, animal models of neuropathic pain have shown increased TSPO expression co-localised with activated microglia and astrocytes (Liu et al., 2016; Wei et al., 2013). Similarly, TSPO expression has been localised with activated astrocyte and microglia in animal models and human investigations of multiple sclerosis, Alzheimer's disease and HIV encephalitis (Abourbeh et al., 2012; Chen and Guilarte, 2006; Cosenza-Nashat et al., 2009; Gulyas et al., 2009; James et al., 2017) and animal models and human post-mortem studies of ischemia (Cosenza-Nashat et al., 2009; Martin et al., 2010; Rojas et al., 2007). Further, TSPO PET imaging in amyotrophic and primary lateral sclerosis patients demonstrates increased TSPO signal in the primary motor cortex (Alshikho et al., 2016; Alshikho et al., 2018; Paganoni et al., 2018; Zurcher et al., 2015), a region where glial activation can be documented histologically (Hudson et al., 1993; Kawamata et al., 1992; Rothstein et al., 1995). Similarly, in Alzheimer's Disease, glial activation can be observed in amyloid positive regions (Araujo and Cotman, 1992; Rozemuller et al., 1989), and these regions have shown elevated TSPO expression (Kreisl et al., 2013; Parbo et al., 2017). Likewise, glial activation has been reported in the basal ganglia of Huntington's Disease patients (O'Kusky et al., 1999), who also have shown elevated TSPO expression (Lois et al., 2018). These observations support the use of TSPO as a marker of glial activation. Because in the CNS TSPO can be upregulated by microglia and/or astrocytes (Beckers et al., 2018; Lavisse et al., 2012), our study does not directly allow clarification of which glial cell subtype might contribute to the observed signal. For instance, several animal models have shown that initial upregulation of TSPO might be driven by microglia, whereas astrocytic TSPO upregulation might be maintained throughout the course of the disease (Chen et al., 2014; Chen and Guilarte, 2006; Kuhlmann and Guilarte, 2000; Liu et al., 2014; Martin et al., 2010). This phase-dependent activation of glial cells is supported by human post-mortem studies of multiple sclerosis, showing that in acute lesions, microglia and macrophages are the major cell contributors to TSPO

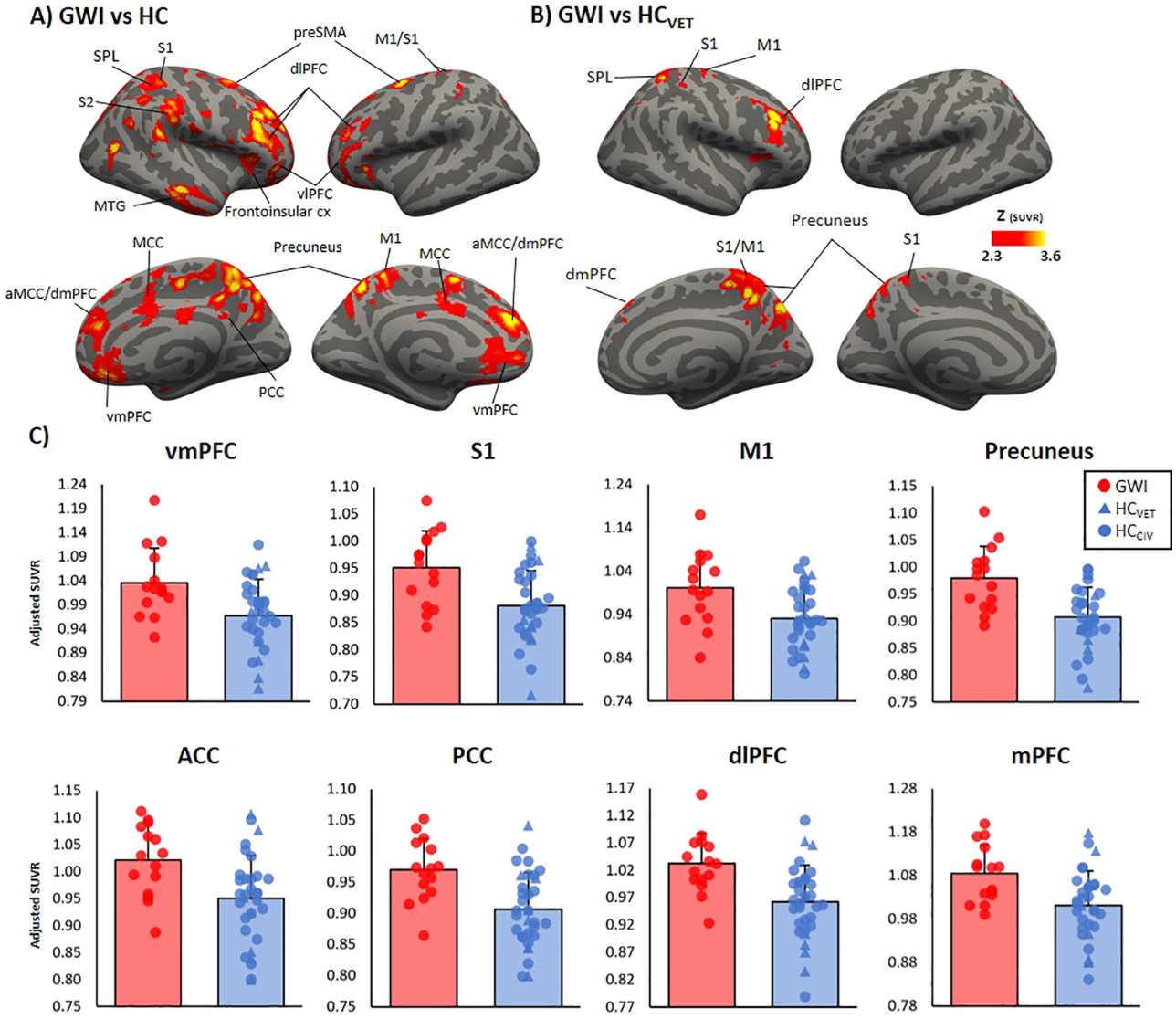

Fig. 2. Voxel-wise group difference in [^{11}C]PBR28 standardized volume uptake (SUVR). A. Surface projection maps displaying areas with significantly elevated [^{11}C]PBR28 SUVR in GWI (n = 15) compared to HC (n = 33), in voxel-wise analyses, data adjusted for sex and genotype. B. Surface projection maps displaying areas with significantly elevated [^{11}C]PBR28 SUVR in GWI (n = 15) compared to HC$_{VET}$ (n = 8), in voxel-wise analyses, data adjusted for genotype. C. Average ± standard deviation SUVR extracted from several clusters identified as statistically significant in the voxel-wise SUVR analysis from A. Data plots have been adjusted for sex and genotype. vmPFC = ventral medial prefrontal cortex; S1 = primary somatosensory cortex; M1 = primary motor cortex; ACC = anterior cingulate cortex; PCC = posterior cingulate cortex; dlPFC = dorsolateral prefrontal cortex; dmPFC = dorsomedial prefrontal cortex; mPFC = medial prefrontal cortex; aMCC = anterior mid cingulate cortex; SPL = superior parietal cortex; MCC = mid cingulate cortex; vlPFC = ventrolateral prefrontal cortex; S2 = secondary somatosensory cortex; cx = cortex.

expression, whereas in chronic lesions, astrocytes are the major contributors to TSPO expression (Cosenza-Nashat et al., 2009). However, because GWI is accompanied by sickness behavior (O'Callaghan and Miller, 2019), and given that microglia are largely the source of neuroinflammatory mediators that underlie sickness behaviors (Dantzer et al., 2008; Konsman et al., 2002; Maier, 2003; Watkins et al., 2007b), it seems likely that microglial activation would account for a significant proportion of the neuroinflammatory signal observed. In addition, several preclinical GWI studies implicate microglia and not astrocytes in neuroinflammation (Carreras et al., 2018; Locker et al., 2017). Furthermore, a recent study employing a dual-ligand approach suggests that in fibromyalgia, a chronic condition that shares clinical features with GWI, the TSPO signal might be indeed driven by microglia rather than astrocytes. In that study, we reported significant elevations in TSPO signal (which may reflect microglial or astrocytic contributions), but not in MAO-B signal [which is thought to reflect mostly astrocytic, but not microglial, contributions; (Albrecht et al., 2019)]. Similar approaches will need to be implemented to understand whether a similar interpretation can apply to GWI as well.

It is important to also stress that, in addition to being upregulated by glial cells within the CNS, TSPO is also highly expressed in activated macrophages and other peripheral immune cells (Lacor et al., 1996). For instance, a recent study has shown elevated TSPO expression in activated macrophages, fibroblast-like synoviocytes and CD4 + T lymphocytes in the synovial tissue of patients with rheumatoid arthritis (Narayan et al., 2018). In physiological conditions, the blood brain barrier (BBB) typically acts as a restriction to prevent easy recruitment into the CNS parenchyma of cells involved in the adaptive immunity response (with the exception of activated T cells) such as leukocytes (Ransohoff and Brown, 2012). However, several preclinical models of GWI document the presence of BBB disruptions (e.g., Abdel-Rahman et al., 2002). Indeed, a study in GW veterans detected CNS

Table 3
GWI [^{11}C]PBR28 correlations with clinical and cytokine variables.

GWI [^{11}C]PBR28 correlations with clinical variables									
		vmPFC	S1	M1	Precuneus	ACC	PCC	dlPFC	mPFC
Kansas GWI score	r value	−0.3772	−0.1244	−0.3110	−0.1458	−0.1728	−0.2780	−0.3772	−0.2967
	p value	0.1840	0.6720	0.2790	0.6190	0.5550	0.3360	0.1900	0.3030
ACR Fibromyalgia score	r value	−0.3472	0.2181	−0.1311	0.1828	0.1263	0.0116	−0.0823	−0.0474
	p value	0.2240	0.4540	0.6550	0.5320	0.6670	0.9690	0.7800	0.8720
ACR Fatigue score	r value	−0.2340	0.2363	−0.0427	0.1678	0.1778	−0.1055	0.0398	0.0858
	p value	0.4210	0.4160	0.8850	0.5660	0.5430	0.7200	0.8930	0.7700
Conner's CPT3 Hit Reaction Time	r value	0.0180	−0.0412	−0.0782	−0.0529	0.0350	0.1562	−0.3173	0.0941
	p value	0.9510	0.8890	0.7910	0.8570	0.9050	0.5940	0.2690	0.7490
BPI Pain Intensity	r value	−0.4269	0.2825	−0.0211	0.2466	0.4259	−0.0009	0.3951	0.1422
	p value	0.1280	0.3280	0.9430	0.3950	0.1290	0.9980	0.1620	0.6280
BDI	r value	−0.2185	−0.0712	−0.1650	−0.0981	−0.2404	−0.2495	−0.2776	−0.3849
	p value	0.4530	0.8090	0.5730	0.7390	0.4080	0.3900	0.3370	0.1740
IL-6	r value	0.1123	0.3274	0.1033	0.3788	0.0386	0.0031	0.0735	0.1925
	p value	0.7150	0.2750	0.7370	0.2020	0.9000	0.9920	0.8110	0.5290
TNF-α	r value	0.2558	−0.4102	−0.2225	−0.2746	−0.3007	−0.1644	−0.2227	0.0674
	p value	0.4480	0.210	0.5110	0.4140	0.3690	0.6290	0.5100	0.8440
IL-1β	r value	0.1495	0.5665	0.1905	0.5963	0.3202	0.1546	0.2831	0.3949
	p value	0.6800	0.0880	0.5980	0.0690	0.3670	0.6700	0.4280	0.2590

autoantibodies to glial fibrillary acidic protein, myelin basic protein, tau, tubulin and other neuro-glial proteins in the peripheral blood that would not be in circulation without at least prior BBB compromise at some point (Abou-Donia et al., 2017). Because the disruption in BBB permeability increases the likelihood of the CNS being infiltrated by activated macrophages or other cell types (Lopes Pinheiro et al., 2016), and given that TSPO is highly expressed in these cells, it is possible that the elevations in the brain levels of TSPO observed in this investigation might in part be due to this phenomenon. The recruitment of peripheral immune cells into the CNS was shown to be able to damage neuronal cells (Ransohoff and Brown, 2012), and thus might contribute to some of the symptoms of GWI such as cognitive/affective decrements and memory loss (Janulewicz et al., 2017; Jeffrey et al., 2019; Sullivan et al., 2018; Sullivan et al., 2003). However, future studies will need to directly measure BBB damage in veterans with GWI to assess the relevance of this mechanism to neuroinflammation.

In the present study, the brain TSPO PET signal did not correlate with circulating levels of proinflammatory cytokines, and when compared to HC, cytokine levels in GWI were not elevated. These results agree with several prior studies in patients with major depression (Richards et al., 2018; Setiawan et al., 2015), seasonal allergy (Tamm et al., 2018), schizophrenia (Coughlin et al., 2016) and in healthy participants imaged after administration of lipopolysaccharide (a potent immune activator) (Sandiego et al., 2015), which also reported no statistically significant correlations between brain TSPO signal and the majority of the peripheral markers of inflammation (although a prior study from our group did report a weak negative correlation between TSPO signal and IL-6 in chronic low back pain [Loggia et al., 2015]).

Why some veterans develop GWI while others do not is still yet to be answered. It is possible that the veterans with GWI have had other toxicant exposures or mTBIs that have 'primed' their glial cells for neuroinflammation at the exposure of further neurotoxicants and stressors (Blaylock and Maroon, 2011; Perry et al., 1985; Watkins et al., 2007a). Certainly, a larger sample size of veterans, ideally with detailed information about other brain insults including mTBIs, life stressors and the types of neurotoxicant exposures, would be required to begin answering some of these questions.

In conclusion, this study is the first to document an elevation of the neuroinflammatory glial marker, TSPO, in the brain of veterans with GWI. Further studies are required to validate and further refine these findings, and to determine whether glial modulation may be a viable therapy for GWI.

Acknowledgments

The study was supported by the following funding sources: DoD-W81XWH-14-1-0543 (MLL), International Association for the Study of Pain Early Career Award (MLL), R01-NS094306-01A1 (MLL), R01-NS095937-01A1 (MLL), R21-NS087472-01A1 (MLL), R01-AR064367 (VN, RRE), R01-AT007550 (VN), W81XWH-18-1-0549 (KS), Martinos Center Pilot Grant for Postdoctoral Fellows (DSA), Harvard Catalyst Advance Imaging Pilot Grant (JMH), P41RR14075, 5T32EB13180 (T32 supporting DSA), and P41EB015896. The authors thank Dr Norman Taylor and Dr Patricia Bachiller for assistance with data collection. The also thank Virginie Esain from Meso-Scale Discovery for her help with the cytokine analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2020.01.020.

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Using Plasma Autoantibodies of Central Nervous System Proteins to Distinguish Veterans with Gulf War Illness from Healthy and Symptomatic Controls

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Received: 19 July 2020; Accepted: 1 September 2020; Published: 5 September 2020

Abstract: For the past 30 years, there has been a lack of objective tools for diagnosing Gulf War Illness (GWI), which is largely characterized by central nervous system (CNS) symptoms emerging from 1991 Gulf War (GW) veterans. In a recent preliminary study, we reported the presence of autoantibodies against CNS proteins in the blood of veterans with GWI, suggesting a potential objective biomarker for the disorder. Now, we report the results of a larger, confirmatory study of these objective biomarkers in 171 veterans with GWI compared to 60 healthy GW veteran controls and 85 symptomatic civilian controls (n = 50 myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) and n = 35 irritable bowel syndrome (IBS)). Specifically, we compared plasma markers of CNS autoantibodies for diagnostic characteristics of the four groups (GWI, GW controls, ME/CFS, IBS). For veterans with GWI, the results showed statistically increased levels of nine of the ten autoantibodies against neuronal "tubulin, neurofilament protein (NFP), Microtubule Associated Protein-2 (MAP-2), Microtubule Associated Protein-Tau (Tau), alpha synuclein (α-syn), calcium calmodulin kinase II (CaMKII)" and glial proteins "Glial Fibrillary Acidic Protein (GFAP), Myelin Associated Glycoprotein (MAG), Myelin Basic Protein (MBP), S100B" compared to healthy GW controls as well as civilians with ME/CFS and IBS. Next, we summed all of the means of the CNS autoantibodies for each group into a new index score called the Neurodegeneration Index (NDI). The NDI was calculated for each tested group and showed veterans with GWI had statistically significantly higher NDI values than all three control groups. The present study confirmed the utility of the use of plasma autoantibodies for CNS proteins to distinguish among veterans with GWI and other healthy and symptomatic control groups.

Keywords: etiology; Gulf War Illness; CNS autoantibodies; myalgic encephalomyelitis/chronic fatigue syndrome; irritable bowel syndrome

1. Introduction

Although the 1991 Gulf War (GW) only had less than two months of air strikes and less than a week of ground combat, approximately one-third of the 697,000 U.S. veterans developed a combination of health symptom complaints, including debilitating fatigue, chronic headache and body pain, memory and concentration difficulties, gastrointestinal problems, and skin abnormalities, known as Gulf War illness (GWI) [1–3]. In addition, some GW veterans also had increased rates of two other distinct conditions, Irritable Bowel Syndrome (IBS) and Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS), which have overlapping symptoms with GWI [4,5]. Given these overlapping symptoms with other chronic conditions, it has been difficult to confirm the presence or absence of GWI or to differentiate between these disorders [6–8]. Neuroimaging has been found to be useful as a differential diagnostic tool for GW ill versus GW healthy individuals; however, what has been missing is the ability to use a less invasive, more readily available, and less costly tool, such as blood biomarkers to differentiate GWI from GW healthy status and differentiate GWI from another chronic multisymptom discord [6,8]. There have been some encouraging blood biomarker studies reporting differences between GWI cases and controls on neuroinflammatory markers that require validation in other GWI cohorts [9–11]. In addition, recently, we reported on a pilot study of serum biomarkers, which found seven out of eight markers significantly differed in veterans with GWI versus symptomatic controls with lower back pain, suggesting new potential blood markers for GWI [12]. The current study expanded on these prior findings by adding newly developed cutting-edge blood plasma autoantibodies in GW veteran and civilian cohorts, including those with IBS and ME/CFS, to identify whether veterans with GWI have the signature central nervous system (CNS) damage associated with their deployment that is different from other groups with overlapping chronic symptoms. IBS is a chronic mutisymptom illness that affects the gastrointestinal system and results in diarrhea or constipation or both. Although the cause of IBS is not known, it may result from altered gut motility, stress, environmental exposures, and genetic predisposition. It has also been shown to be related to alterations of the gut–brain axis in animal models of GWI and in a pilot study of GW veterans [13–16]. ME/CFS is characterized by extreme fatigue, muscle pain, headaches, multijoint and throat pain, lymph node swelling and soreness, chronic insomnia, and sleep disorders [6]. In addition, it may cause loss of memory and reduced concentration. Contributing factors may include viruses, weakened immune system, stress, or environmental exposures. Although some symptoms among the three disorders (GWI, IBS, ME/CFS) overlap, the etiologies differ, raising the question whether objective blood markers of GWI could be distinguished among these other chronic medical conditions.

GW veterans were exposed to numerous environmental neurotoxicants, including acetylcholinesterase (AChE)-inhibiting organophosphate pesticides and nerve gas agents [17,18]. Early studies investigated the hypothesis that GWI resulted from combined exposures of GW-relevant toxicants including pyridostigmine bromide (PB), *N*,*N*-diethyl-meta-toluamide (DEET), permethrin, and chlorpyrifos in hens [19,20]. Mixed exposures to multiple toxicants resulted in significantly greater toxic effects than separate exposures. More recent results with GW veterans who were pesticide applicators during the war also showed that combination exposures to PB and pesticides were associated with higher rates of GWI and specifically, with diminished CNS functioning on mood and cognition [18].

Pesticides used during the GW easily enter through the blood–brain barrier (BBB) because they are lipid-soluble [19,20]. These neurotoxicants have been found to be associated with autoantibodies to CNS proteins in the blood in several prior studies and exposed groups [12,20,21]. These exposures have been associated with neurological symptoms associated with CNS cellular functioning. For example, studies showed increased levels of CNS cellular proteins in pesticide-exposed participants with neurological symptoms [22–24]. These results were similar to those found in our pilot study of ill GW veterans [12].

The brain has two types of cells: neurons and supporting glial cells, including astrocytes and oligodendrocytes [21,25–27]. Neurons are characterized by the cell body and two additional parts,

including axons and dendrites. Proteins in the axon include neurofilament triplet proteins (NFP), tubulin, tau, calcium/calmodulin kinase II (CaMKII), and (α-syn) [20,21,26,28]. Proteins in the dendrites include microtubule associate protein (MAP-2) [28]. Microtubules and tau make up the cytoskeleton of neurons.

Glial support cells include oligodendrocytes that myelinate axons using myelin basic protein (MBP) and myelin-associated glycoprotein (MAG). Astrocytes secrete glial fibrillary acidic protein (GFAP) and S100B only in the CNS [21,29,30].

The present study was carried out to use our newly developed biomarker test to differentiate GWI from other chronic conditions and healthy controls and confirm/validate our previous preliminary report of a small number of GW veterans and symptomatic controls showing increased CNS protein autoantibodies in their blood [12]. We hypothesized that as a result of neurotoxicant exposures during the war, autoantibodies to these ten CNS proteins would be increased in veterans with GWI when compared with other healthy and symptomatic control groups. Specifically, we hypothesized that neuronal and glial CNS proteins would differ in veterans with GWI compared with healthy and symptomatic controls with similar multisymptom disorders, including IBS and ME/CFS.

2. Materials and Methods

Study Population: GW illness consortium (GWIC) and the Dynamic Modeling of GWI study participants, two Department of Defense supported studies at Boston University and Nova Southeastern University, provided plasma samples from veterans deployed to the 1991 GW. Additional GWI participant samples were shared from the New England School of Acupuncture. These three established biorepositories of GW veterans were used from veterans who consented to share their blood samples for future studies. Control samples were provided by the Congressionally Directed Medical Research Program (CDMRP) funded studies in Boston and Florida, and samples from patients with Irritable Bowel Syndrome came from the biorepository at Beth Israel Deaconess Medical Center. Institutional Review Boards (IRBs) approvals from these biorepositories were obtained from Boston University, Nova Southeastern University, the Miami VA Medical Center, and Beth Israel Deaconess Medical Center.

The same standard operating procedures for phlebotomy, plasma separation, aliquoting, and storage were followed by all labs for all samples. Plasma samples were obtained from fasting subjects. Samples remained frozen at −80 °C until shipped for autoantibody analysis.

Cases and controls were determined by Kansas GWI criteria [31]. This criterion requires GW veterans to self-report symptoms in 3 out of 6 symptom domains (neurologic/mood/cognitive, fatigue, pain, gastrointestinal, respiratory, and skin). Veteran controls were deployed to the GW and did not meet the Kansas GWI or exclusionary criteria. Exclusions included CNS medical conditions and psychiatric illnesses that could account for their symptoms [31]. Plasma samples from symptomatic controls came from prior studies of individuals with ME/CFS and IBS. ME/CFS cases were determined by using 1994 CDC criteria [5]. IBS cases were determined by Rome III criteria [32]. The full cohorts have been described in previous papers (GWIC, ME/CFS, IBS, GWIC subsample) [4,12,14,33,34]. Institutional review boards at Nova Southeastern University/Miami VA Medical Center, New England School of Acupuncture, Beth Israel Deaconess Medical Center, and Boston University provided approval. All participants signed consent to use their plasma for follow-up studies of GWI biomarkers.

Ethical Statement: Approval for the use of stored blood samples for this study was obtained from the Duke University Health System Institutional Review Board for Clinical Investigations on 9 October 2017 and from the Boston University Medical Campus Institutional Review Board on 19 January 2018. The specific protocol components for Duke University were: Protocol ID: Pro00003202, Reference ID: 335940, Principal Investigator: Mohamed Abou Donia, Protocol Title: 'Nervous System Injury'. The specific protocol components for Boston University were Protocol ID: H-34334, Reference ID: 1288716, Principal Investigator: Kimberly Sullivan, Protocol Title: 'Novel Autoantibody Serum and Cerebrospinal Fluid Biomarkers in Veterans with Gulf War Illness'.

2.1. Materials

The proteins used in this study were as follows: Tubulin (human recombinant, Cat. #PRO-982, ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA), Microtubule-Associated Protein 2 (MAP-2, human recombinant, Cat. #TP316775, OriGene, Rockville, MD, USA), Tau-381 (human recombinant, Cat. #AG952, MilliporeSigma, Burlington, MA, USA), Neurofilament Protein (NFP, Cat #PRO-523, ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA), Calmodulin Kinase II (human recombinant, CaMKII, Cat #H000000H15-P01, Novus Biologicals, Littleton, CO, USA), Alpha-synuclein (human recombinant, Cat. #AS-55555, AnaSpec, Fremont, CA, USA), Myelin Basic Protein (MBP, human, Cat. #30R-AM030, Fitzgerald Industries International, Acton, MA, USA), Myelin-Associated Glycoprotein (MAG, human recombinant, Cat. #131–86-H02H, Sino Biological Inc., Wayne, PA, USA), Glial Fibrillary Acidic Protein (GFAP, human, Cat. #345996, CalBiochem, San Diego, CA, USA), and S100B Protein (human, Cat. #30R-AS002, MilliporeSigma, Burlington, MA, USA).

2.2. Procedures

Plasma procedures: All sites used the same written standard operating procedures for venipuncture, blood handling, plasma separation, aliquoting, and storage at −80 °C. Blood samples were collected prior to intervention for treatment trials. Samples remained frozen until sent for analyses and were visually inspected to not have hemolysis.

Western blot assay: In this study, a Western blot analysis was used for determination of CNS autoantibodies and antigens from the plasma samples of GWI cases and healthy and symptomatic controls. All plasma samples were analyzed three times for consistency and followed the protocol previously published in [12]. Specifically, each CNS protein was loaded into 10 ng/lanes. Immunoglobulin G (IgG) was loaded into a 100 ng/lane. All proteins were denatured and electrophoresed on SDS-PAGE (gradient 4% to 20% gradient) and a separate gel was used for each plasma sample. Enhanced chemiluminescence was used to determine if proteins were found by using a Typhoon 8600 variable model recorder (GE Lifesciences, Marlborough, MA, USA). The signal intensity was determined by Bio-Rad Quantity One image analysis software (Hercules, CA, USA). Specifically, the protein bands were quantified on digitized images in the mid-dynamic range using Quantity One software (Bio-Rad) and densitometry measurements were normalized to IgG in the same samples. Lab researchers were blinded to the case–control status of the samples.

2.3. Calculations

Measurement of chemiluminescent optical density for cases and controls was obtained by dividing plasma IgG concentrations. This optical density measure was normalized to controls and expressed as fold-change from healthy controls. Therefore, the CNS autoantibody measurements were presented as mean triplicate assay values normalized to healthy control values.

2.4. Neurodegeneration Index (NDI)

This new index was designed to determine the overall neurodegenerative condition of an individual based on the level of autoantibodies in the plasma. It is calculated by adding all of the values of autoantibodies for each neural protein, and then, dividing the sum by the number of autoantibodies used. Finally, this value is multiplied by 10 to produce the NDI.

$$\text{Neurodegeneration Index (NDI)} = (\text{The Sum of Autoantibodies to “n” Proteins/n}) \times 10 \quad (1)$$

The NDI is used here as a simple, blood-based proxy to determine the extent of neurodegeneration of an individual, based on a plasma assay of autoantibodies for an individual.

2.5. Statistical Methodology

Descriptive statistics are presented as mean ± SE for continuous variables and as number and percent of participants per category of categorical variables. Subjects' demographic values were compared across the four groups using one-way analysis of variance for continuous outcomes and the chi-square test for categorical outcomes. Mean values of the antibodies were compared across groups using analysis of covariance (ANCOVA) adjusting for age, sex, and race. p values were two-sided. To account for multiple comparisons, $p < 0.001$ was accepted as statistically significant for the comparisons between treatments on antibody levels. Analyses were conducted using SAS Version 9.4 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Specificity of Serum Autoantibodies

The specificity of the serum autoantibodies against all tested neural proteins was previously reported by performing protein/peptide competitive assay [12,26]. The specificity of an autoantibody in the sera was assessed by performing a peptide/antigen absorption assay by preabsorbing the serum with the target proteins. The preabsorbed serum was tested by Western blot (Figures 1–3).

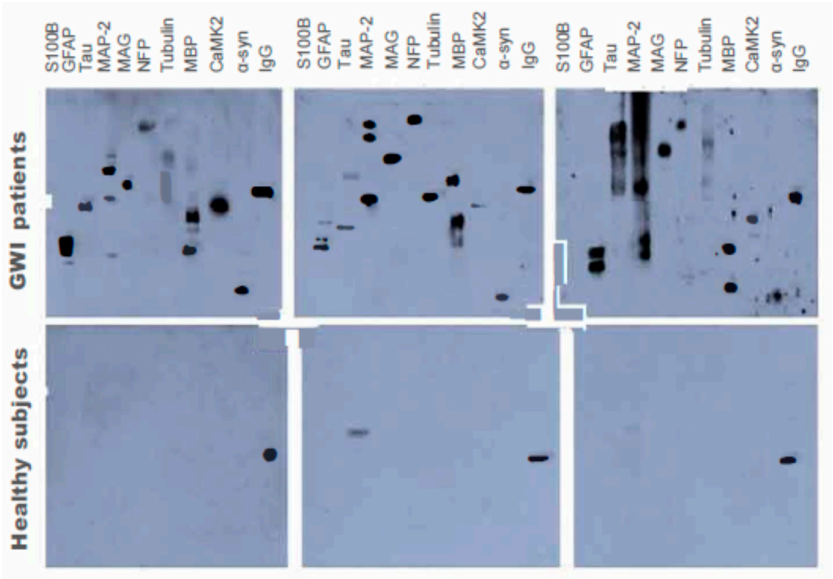

Figure 1. Representative panel of Western blotting from three cases of the GWI patients (upper panels), and healthy controls (lower panels).

3.2. Participant Demographics

Demographics are presented in Table 1. Participants were 175 veterans with GWI, 60 GW veteran healthy controls, 37 IBS controls, and 50 ME/CFS symptomatic controls. Significant differences were seen for age, sex, and race.

This study was carried out to use our newly developed neurodegenerative biomarkers to diagnose veterans of the 1991 Gulf War with GWI compared with healthy and symptomatic controls. The biomarkers consist of circulating autoantibodies of ten neural proteins (six neuronal and four glial) determined in the plasma of GW veterans with GWI, healthy Gulf war veterans, veterans with ME/CFS, and IBS that were used as controls. The NDI was calculated as described in the methods above and was assessed among the groups by chi-square tests.

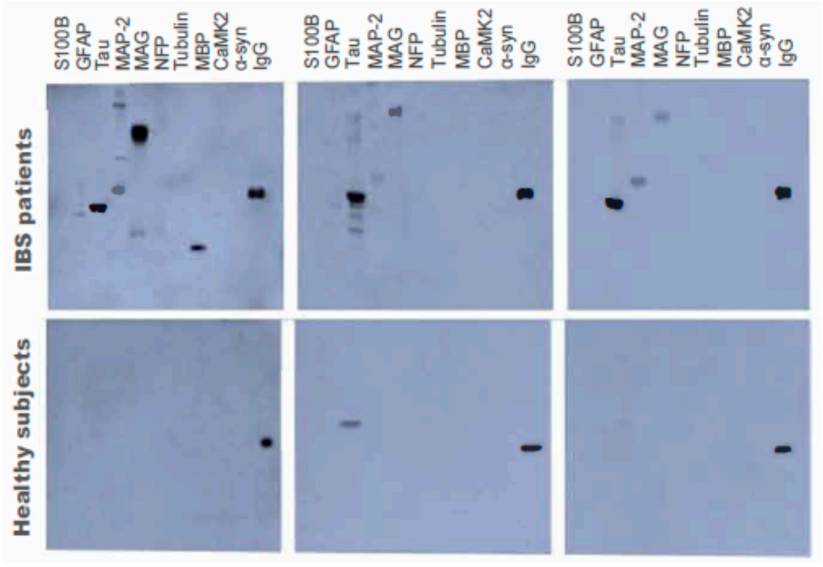

Figure 2. Representative panel of Western blotting from three cases of the IBS patients (upper panels), and healthy controls (lower panels).

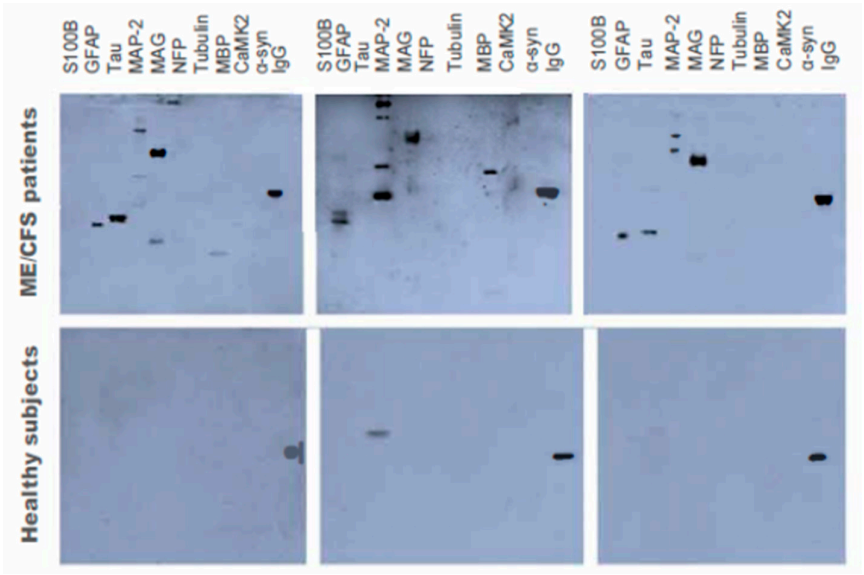

Figure 3. Representative panel of Western blotting from three cases of the ME/CFS patients (upper panels), and healthy controls (lower panels).

3.3. Autoantibody Levels for Neuronal and Glial Proteins using Western Blot

Autoantibodies were determined for GWI cases, GW healthy veteran controls, IBS symptomatic controls, and ME/CFS symptomatic controls for the six neuronal proteins: NFP, tubulin, tau, MAP-2, CaMKII, and α-syn. In addition, four proteins from two types of glial cells were measured, including MBP and MAG from oligodendrocytes and GFAP and S100B from astrocytes.

The first analysis, which compared all three control groups combined (GW controls, IBS and ME/CFS groups), with veterans with GWI showed significantly increased mean levels for veterans GWI for nine out of the ten autoantibodies (Table 2; Figure 4). The only exception was for the glial protein S100B, whose mean level was similar to that of healthy controls (Table 2). In addition, there were no interacting the groups for any of the outcome measures.

Table 1. Demographic information of Gulf War Illness cases and healthy Gulf War and symptomatic controls.

Data	GWI Cases	GW Healthy Controls	IBS Controls	ME/CFS Controls
N	175	60	37	50
Age * (Mean ± SD)	48.7 ± 7.8	50.92 ± 7.48	39.40 ± 13.93	46.74 ± 10.24
Sex *				
Male (%)	80.2%	93.3%	8.6%	10%
Female (%)	19.8%	6.7%	91.4%	90%
Race *				
Caucasian (%)	81.3%	73.7%	85.7%	91.3%
African American (%)	12.9%	23%	8.6%	8.7%
Other/Multiracial (%)	5.8%	3.3%	5.7%	0.0%

Note: * denotes significant differences $p < 0.01$ across the four groups. We obtained information for age, sex, and race from 171 individuals for GWI veterans, and 35 individuals for IBS controls.

Table 2. Autoantibodies against neural proteins in GWI cases and healthy and symptomatic controls [a] using ANCOVA analysis and adjusting for age, sex, and race.

		GWI vs. All Controls	GWI vs. GW Controls	GWI vs. IBS Controls	GWI vs. ME/CFS Controls
A. Neuronal Proteins					
Neurofilament Triplet Proteins (NFP)	Mean (SE)	3.42 (0.19) ***	1.88 (0.26) ***	0.86 (0.02) ***	1.18 (0.05) ***
	Range	2.6–15.15	0.16–9.74	0.64–1.23	0.51–2.25
Tubulin	Mean (SE)	4.13 (0.25) ***	2.36 (0.30) ***	1.14 (0.03) ***	2.71 (0.24) **
	Range	0.32–15.15	0.09–10.76	0.62–15.36	0.63–7.39
Microtubule Associated Protein Tau (Tau)	Mean (SE)	2.92 (0.22) ***	1.57 (0.17) ***	1.24 (0.13) ***	1.02 (0.07) ***
	Range	0.33–10.55	0.34–5.13	0.52–4.11	0.40–3.36
Microtubule Associated Protein-2 (MAP-2)	Mean (SE)	9.66 (0.73) ***	5.04 (0.76) ***	1.05 (0.07) ***	6.97 (0.35) **
	Range	0.88–27.42	0.24–23.00	0.64–2.45	1.40–14.02
Calcium/Calmodulin Kinase 2 (CaMKII)	Mean (SE)	2.04 (0.15) ***	1.20 (0.13) ***	0.70 (0.03) ***	1.16 (0.05) ***
	Range	0.10–5.50	0.15–4.50	0.37–1.43	1.11–1.92
Alpha Synuclein (α-syn)	Mean (SE)	2.52 (0.19) ***	1.46 (0.02) ***	0.78 (0.06) ***	1.13 (0.05) ***
	Range	0.17–11.77	0.37–6.45	0.51–1.93	0.41–1.93
B. Glial Proteins: Oligodendrocytes					
Myelin Basic Protein (MBP)	Mean (SE)	4.28 (0.18) ***	2.17 (0.32) ***	1.19 (0.03) ***	1.52 (0.09) ***
	Range	0.09–17.34	0.42–11.83	0.74–1.7	0.44–4.33
Myelin Associated Glycoprotein (MAG)	Mean (SE)	4.94 (0.28) ***	2.12 (0.25) ***	3.20 (0.21) *	1.58 (0.11) ***
	Range	0.24–20.94	0.38–6.51	1.03–6.51	0.26–4/48
Glial Proteins: Astrocytes					
Glial Fibrillary Associated Protein (GFAP)	Mean (SE)	4.27 (0.18) ***	2.34 (0.30) ***	0.84 (0.03) ***	4.86 (0.26)
	Range	0.39–13.14	0.35–14.98	0.45–1.38	0.66–8.44
Glial S100B (S100B)	Mean (SE)	1.17 (0.04)	1.16 (0.03)	0.95 (0.03)	1.34 (0.06) *
	Range	2.60–2.41	0.37–2.74	0.53–1.34	0.49–2.26

Note: * $p < 0.01$ ** $p < 0.001$ *** $p < 0.0001$. [a] Values reflect fold change relative to control.

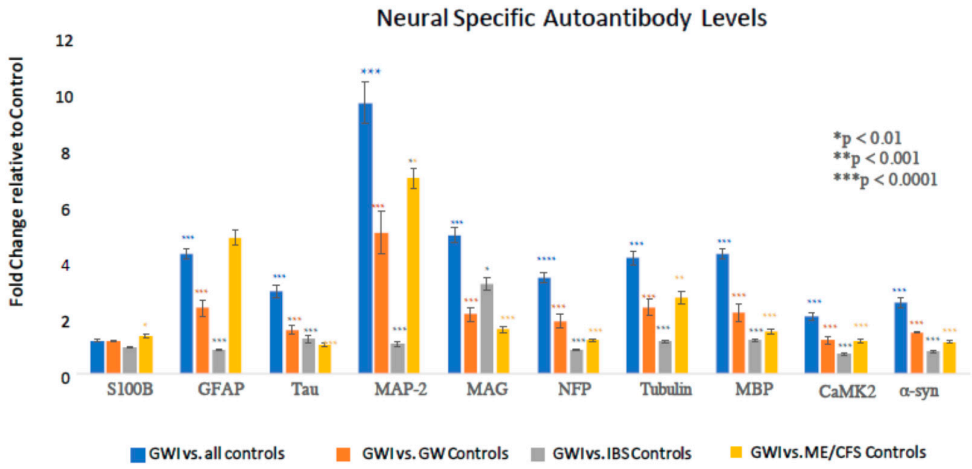

Figure 4. Neural autoantibodies in plasma of GWI cases, GW healthy controls, IBS controls, and ME/CFS controls. *** $p < 0.0001$ (blue) GWI group to all three control groups combined using ANCOVA adjusted for age, sex, and race. *** $p < 0.0001$ (orange) GWI group to GW veteran control group using ANCOVA adjusting for age, sex, and race. *** $p < 0.0001$ (grey) GWI group to IBS group using ANCOVA adjusting for age, sex, and race. *** $p < 0.0001$ (yellow) GWI group to ME/CFS group using ANCOVA adjusting for age, sex, and race.

The next analysis compared only GWI cases to GW veteran controls. The results of this comparison also showed that mean levels of nine out of the ten plasma autoantibodies of GWI cases were significantly higher for veterans with GWI than for healthy GW controls. Again, levels of S100B autoantibodies for GWI cases were not significantly increased from GW veteran controls.

Next, we compared GWI cases to symptomatic non-veteran IBS controls. The results again showed higher mean levels of nine out of ten autoantibodies for GWI cases compared to IBS controls. Again, the only non-significantly different autoantibody was S100B (Table 2).

Finally, we compared GWI cases to symptomatic non-veteran ME/CFS controls. The results showed higher mean levels of nine out of ten autoantibodies for GWI cases compared to ME/CFS controls. The only non-significantly different autoantibody between the two groups was for the GFAP protein (Table 2).

3.4. Neurodegeneration Index (NDI)

The Neurodegeneration Index score was calculated as described above for each tested group and the results were as follows (Figure 5): GWI = 39.35, GW healthy controls = 21.3, IBS controls = 11.94, and ME/CFS controls = 23.47. The mean NDI score for veterans with GWI was significantly higher than in all controls combined ($p < 0.0001$). In addition, the percentage of participants with NDI > 20 was significantly higher in GWI cases than in all controls combined (94.3% vs. 44.2%; $p < 0.0001$ via the chi-square test). The percentage of participants with NDI > 30 was also significantly higher in GWI cases than in all controls combined (71.8% vs. 14.3%; $p < 0.0001$ via the chi-square test).

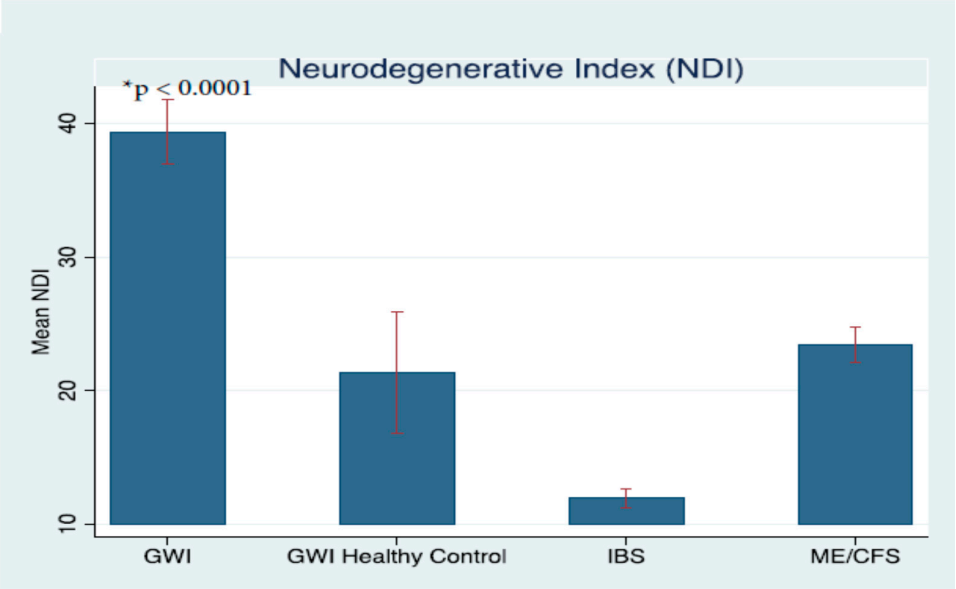

Figure 5. Neurodegenerative index score for GWI cases, GW healthy controls, IBS controls, and ME/CFS controls. Error bars represent the standard error of the mean. The mean NDI for GWI participants is significantly higher than in all controls combined ($p < 0.0001$).

4. Discussion

For the past 30 years, there has not been an objective diagnostic marker for GWI, which has hindered research in the field. Chronic symptoms reported by veterans with GWI have included headache, memory and attention decrements, debilitating fatigue, chronic pain, and gastrointestinal problems [2,3,35]. Many of these symptoms overlap with other comorbid conditions, including IBS and ME/CFS, necessitating the need for an objective marker that can delineate among these diagnostic groups. The present well-powered study confirms and expands the results of our previous descriptive study, where we identified a unique signature of objective biomarkers of CNS proteins in blood samples of 20 GW veterans compared with 10 controls [12]. In this study, we confirmed our prior results in a much larger sample of over 300 participants, including ill and healthy GW veterans and other symptomatic controls with IBS and ME/CFS. Specifically, we compared GWI cases vs. three control groups (GW controls, IBS, ME/CFS) and found that GWI cases had higher autoantibodies than all combined controls in nine out of ten autoantibodies. We then compared GWI cases with the three control groups separately and also found that the GWI cases showed significantly higher levels of nine out of ten autoantibodies than GW controls, IBS, or ME/CFS groups. These results clearly distinguish GWI cases not only from healthy GW counterparts but also other symptomatic controls with chronic multisymptom disorders.

Our results showed significantly elevated CNS autoantibodies in the plasma of veterans with GWI. The presence of low levels of autoantibodies in the plasma of GW healthy controls is consistent with previous findings in healthy individuals [12,36]. These results suggest that GW healthy controls had no lasting CNS effects from their deployment. In contrast, increased levels of CNS autoantibodies are consistent with veterans with GWI's chronic neurological complaints and thus, provides an objective biomarker of the illness. The results revealed large increases in autoantibodies in the GWI cases compared to all controls except for S100B. These increases were significantly higher than controls with autoantibodies against MAP-2, showing the highest overall level in all groups tested. These were followed by autoantibodies against myelin and other glial-related proteins showing the highest levels (MAG > MBP > GFAP) and then, followed by increased neuronal cytoskeletal protein autoantibodies against Tubulin > NFP > Tau.

Our results show that autoantibodies to neural proteins can be used as biomarkers for diagnosis and prognosis of GWI and may also provide insight into the potential mechanisms of GWI. The only consistent risk factors for GWI are environmental exposures, including the use of pyridostigmine bromide pills and pesticides, which are now known to adversely affect the CNS in significant or combined dosages [14,15,17–20,24–28]. Although a total of over 50 pesticide products were used during the Gulf War, less than 20 were designated as "pesticides of concern" by the Department of Defense, including the insecticides permethrin and lindane as well as the repellant, DEET (*N*,*N*-diethyl-*m*-toluamide) and organophosphate insecticides and nerve gases, sarin and cyclosarin [23]. These same exposures were recently shown by our group to be associated with higher rates of GWI and specifically, with worse mood and cognitive functioning [18].

Furthermore, several studies have shown some organophosphorus (OP) compounds, such as sarin and chlorpyrifos in addition to inhibiting acetylcholinesterase, also cause neurodegeneration of the CNS [37–40]. A recent study reported increased CNS autoantibodies in blood from farm workers exposed to OP pesticides [41]. Investigations into the mechanisms by which these compounds cause neurodegeneration have established that OPs increase the activity and expression of CaMKII, which causes hyperphosphorylation of neural proteins, leading to their aggregation and slowing of axonal transport, resulting in neuronal cell death [19,42–45]. In agreement with this is our prior finding that airline crews who were exposed to OPs developed autoimmune antibodies to neural proteins [46]. Another study using Magnetic Resonance Imaging (MRI) examination of another cohort of aircrews, showed decreased white matter microstructure and blood perfusion are potential causes of cognitive and mood symptoms experienced by the aircrews [46].

These results suggest the involvement of white matter alterations in the development of GWI is consistent with increased autoantibodies against MBP and MAG that are present in myelinated axons [9,37,39,44]. Blood markers of MBP are also elevated in myelin-related CNS disorders. Increased autoantibodies to MBP in the plasma of veterans with GWI correlate with demyelination following axonal degeneration caused by exposure to OPs [12,37,39]. GFAP is a glial protein that is involved in white matter and blood–brain barrier functioning [21]. This finding also correlates with our recent finding that GFAP almost completely distinguished between GWI cases and controls in our prior pilot study [12]. This also correlates with recent findings of increased neuroinflammation seen in imaging the brains of veterans with GWI, as shown by significantly greater glial activation using PET brain imaging [47].

CaMKII is widely distributed in the CNS, constituting up to 2% of the protein in the hippocampus [48]. Exposure to organophosphates, such as di-isopropyl fluorophosphate (DFP), a surrogate compound for sarin, enhanced Ca^{++} release and increased expression and activity of CaMKII, resulting in hyperphosphorylation of several cytoskeletal proteins, i.e., tubulin, MAP-2, Tau, and neurofilament triplet proteins [44,45,49]. Increased phosphorylation of MAP-2, Tubulin, and Tau resulted in their aggregation and slowing of axonal transport [44,45,49]. CaMKII-induced hyperphosphorylation caused significant increase in both c-fos and c-jun expression, leading to apoptosis mediated by cytochrome c released from mitochondria due to the imbalance between the Bax, Bcl-2, and BCl-xl proteins triggered by the generation of Reactive Oxygen Species [29,30].

The results show that autoantibodies against S100B were not different from controls and were consistent with its neuroprotective action and the chronic nature of GWI. S100B's half-life is 2 h in blood, supporting the use of its autoantibodies as biomarkers for neuronal conditions [29,30].

When the results of GWI cases were compared to controls with IBS, autoantibodies values were much higher, which is consistent with the fact that IBS is not considered a neurodegenerative disorder. The only elevated autoantibodies in IBS controls were against MAG but even that was less than half that of GWI cases. These results not only confirm the validity of our test as a biomarker for CNS effects, but also establishes its specificity as a marker for chronic GWI. MAG comes from oligodendrocytes in CNS and by Schwann cells in the periphery. The present results suggest that MAG protein was

released from peripheral nerves in the gastrointestinal tract, a major target for IBS, suggesting its potential use for that disorder.

Furthermore, ME/CFS symptomatic controls exhibited levels of autoantibodies against neural proteins that were intermediate between veterans with GWI and controls with IBS. ME/CFS is characterized by body and muscle pains as well as some CNS symptoms, including debilitating fatigue. GWI cases had higher levels of all autoantibodies except for GFAP when compared with ME/CFS controls. The increased GFAP levels in ME/CFS suggest a potential marker and pathobiology for that disorder. Recent studies from other groups have shown increased antibodies against ß2-adrenergic receptors in ME/CFS patients [50,51]. This suggests that ME/CFS is more similar to GWI than IBS based on these autoantibody biomarkers, but GWI still clearly represents a unique disorder based on different autoantibody patterns.

Increased autoantibodies of biomarkers NFP, tau, tubulin, and MBP, and neuronal cytoskeletal disruptions, including microtubule instability, axonal degeneration, and altered axonal transport, have been found in many cell and animal studies of toxicant-induced models of GWI [27,42–45,49,52–54]. We are only aware of the following prior studies, including our prior pilot study, showing increased autoantibodies in much smaller pilot studies of GW veteran blood samples [12,55–58]. To our knowledge, this is the first large, more definitive study to validate these prior animal, cell, and veteran studies in the blood of ill GW veterans compared with combined and separate healthy and symptomatic comparison groups.

We hypothesized that exposures to chemicals present in the GW theater, such as pesticides and nerve gases, can cause CNS damage and release of CNS autoantibodies through the BBB into blood circulation, where B-lymphocytes produce antibodies to proteins and T cells produce cell-mediated immune responses, and IgG autoantibodies are then made [48,59,60]. Theoretically, IgG autoantibodies can enter through the BBB and disrupt CNS functioning, which could lead to symptoms of GWI [59–61]. Further research is needed to confirm this hypothesis. The results of the NDI analyses, showing GWI cases were three times more likely to have an NDI score of 30 or greater, suggest that these individuals may be at increased risk for early onset of age-related neurodegenerative disorders.

Correspondingly, recent studies have reported increased levels of CNS autoantibodies in blood, from neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD), suggesting the need for further studies of the potential increased risk of these disorders in GW veterans [62–64]. Having these reports, together with the results of the present study, raises concerns regarding the likelihood for veterans with GWI to develop neurodegenerative diseases such as AD and/or PD as they age. These CNS protein biomarkers may be useful for determining who is at risk for these disorders in addition to using them in clinical trials for treatment efficacy of GWI.

Limitations

Like all studies, our study had limitations. GWI diagnosis was based on veterans' self-reported symptoms, which could have introduced some classification errors. In addition, some veterans could have very early signs of neurodegenerative disorders that were not picked up in the clinical evaluations, which could have increased autoantibody levels in the veterans. However, the Kansas criteria for GWI would have excluded known cases of these disorders, including AD, PD, and other chronic illnesses that could have accounted for their chronic symptoms [31]. There were also sex differences within our groups as might be expected, with more women in the ME/CFS and IBS groups and more men in the GWI groups, and although these sex differences were controlled for in the analyses, future studies should more directly compare these autoantibody outcomes by sex. A major strength of our study included the large sample size and the inclusion of both healthy and symptomatic veteran groups in this objective biomarker study. In addition, the CNS autoantibody analyses were similar chronic multisymptom disorders (IBS, ME/CFS). We confirmed and validated our prior preliminary results of increased autoantibodies in a much larger sample of veterans with GWI compared with healthy GW veterans and with symptomatic non-veteran IBS and ME/CFS controls [12]. This study confirmed

that nine of the ten autoantibodies were significantly increased in veterans with GWI, suggesting considerable CNS differences compared to both healthy and symptomatic controls. This confirms our prior studies, which suggested a strong CNS component to GWI [18,38]. The present study confirmed the utility of the use of plasma autoantibodies for CNS proteins to distinguish among veterans with GWI and other healthy and symptomatic control groups and our newly developed NDI summary score can be further utilized to compare pre and post treatment trial efficacy.

Author Contributions: Conceptualization, M.B.A.-D., K.S., J.M., L.A.C., E.K. and N.G.K.; Data curation, E.Q. and D.D.N.; Formal analysis, E.Q., J.L. and J.M.; Funding acquisition, M.B.A.-D. and K.S.; Investigation, M.H.K.; Methodology, M.B.A.-D., E.S.L., J.M., L.A.C., E.K., M.A., N.G.K., D.D.N. and K.S.; Project administration, E.S.L., M.H.K., J.L., L.A.C., E.K., M.A., D.D.N. and K.S.; Resources, M.A. and N.G.K.; Supervision, M.B.A.-D.; K.S.; Writing—original draft, M.B.A.-D. and K.S.; Writing—review & editing, E.S.L., M.H.K., E.Q., J.M., L.A.C., E.K., M.A., N.G.K., D.D.N. and K.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the U.S. Army Medical Research and Materiel Command under contracts No. W81XWH-15-0640, W81XWH-15-0641, W81XWH-15-1-0695, and W81XWH-16-1-0528. ME/CFS samples were collected under RO1 1A1065723 (N Klimas/MA Fletcher co-PIs) Immunologic Mechanisms, Biomarkers, and Subsets in ME/CFS.

Acknowledgments: We thank the veterans who agreed to share their biorepository samples for this study. We would also like to thank Shruti Durape for data entry and data cleaning. We thank Hagir Suliman for technical assistance and Brahma Mulugu for receiving the plasma samples and their storage in the freezer. The views, opinions, and findings contained herein are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation. In the conduct of this research, the investigators adhered to the policies regarding the protection of human studies as prescribed by 45 CFR 46 and 32 CFR 219 (Protection of Human Subjects).

Conflicts of Interest: The authors declare no conflict of interest.

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