DIRECT AND INDIRECT EFFECTS OF GULF WAR ILLNESS-RELATED CHEMICALS ON MICROGLIA ACTIVATION: IN VITRO AND EX VIVO STUDIES

by

ALEXANDRIA ASHLEIGH GOODYEAR

(Under the Direction of Nikolay M. Filipov)

Abstract

Gulf War Illness (GWI) affects approximately 30% of the deployed 700,000 U.S. veterans as a multi-symptomatic disease, including neurological symptoms. Here, we used Gulf War Chemical (GWC) combinations employed in preclinical GWI models to evaluate their effects *in vitro* on microglia. When applied directly to microglia, none of the GWC increased inflammatory cytokine secretion; GWC combinations containing corticosterone were anti-inflammatory. Pre-exposure to the GWC pyridostigmine bromide (PB) and permethrin (PM), but not other GWCs, enhanced lipopolysaccharide-induced inflammation. Similarly, only treatment of microglia with plasma from a chronic PB + PM preclinical GWI model resulted in increased inflammatory cytokines. Together, these data indicate the PB + PM preclinical GWI model combination has direct and indirect microglial priming effects, while the *in vivo* neuroinflammation associated with models containing DFP and corticosterone does not appear to involve humoral peripheral input or direct priming/activation of microglia.

INDEX WORDS: Gulf War Illness, microglia, neuroinflammation, ELISA, plasma, cytokines, *in vitro*, *ex vivo*

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DEDICATION

I would like to dedicate this thesis to my friends and family, who constantly provide me with love and support. To my parents, whose good example and dedication taught me to work hard. Finally, this thesis is also dedicated to my loving husband, whose patience, understanding, and constant provision of caffeinated beverages made the completion of this thesis possible.

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Literature Review

The Persian Gulf War and Gulf War Illness

The Persian Gulf War (1990-1991) was an international conflict that occurred when Kuwait was invaded by Iraq in August of 1990. In response, the United Nations sanctioned military action, and a multinational coalition consisting of almost one million troops from the United States, United Kingdom, France, and several dozen other allied forces deployed to the region. This coalition deployment was dubbed Operation Desert Shield and in January of 1991, Operation Desert Storm begun with six weeks of air conflict. In February, a ground offensive was launched resulting in four days of combat before a ceasefire was declared. By June of 1991, the last of the U.S. troops had returned home. The was considered to be a victory for the U.S. with under 150 conflict related casualties reported (RACGWI, 2014).

Despite the success of the war, troops who had served soon begun to experience problems upon their return home. Within six months of the war's end, reports began to come in from the U.S., as well as allied nations, of troops experiencing similar unusual symptoms. These symptoms included fatigue, cognitive impairment, behavioral changes, gastrointestinal abnormalities, rashes, joint and muscle pain (Kang *et al.*, 2000; Kimono, 2021; White *et al.*, 2016). Initially dubbed Gulf War Syndrome, this disease is now known as Gulf War Illness (GWI) and has been recognized officially by both the Department of Veteran Affairs and the Department of Defense (RACGWI, 2014). GWI presents as a chronic, multi-symptomatic

disease afflicting more than 200,000 – approximately one third – of the U.S. troops deployed during the Persian Gulf War (White *et al.*, 2016). Not only was the U.S. reporting GWI, it was also reported in the U.K., France, Canada, Australia, and Denmark (Kerr, 2015). Initially, the cause of these GWI symptoms was unknown, and it did not match with any war-related illnesses previously reported. In addition, it was noted that deployed troops reported higher rates of symptoms than their non-deployed counterparts (Binns, 2008). This led to the conclusion that GWI might have been a result of exposure to hazardous substances to which Gulf War veterans were exposed.

Etiology of GWI and the Gulf War Chemicals

The exact etiology behind GWI is not known, but it is thought to be a combination of the many substances to which troops were exposed during the war. Some of these factors include war-theater stress, as well as over exposure to chemicals, including pesticides and insect repellents such as permethrin (PM) and N-N,Diethyl-3-methylbenzamide (DEET), which were used in excess by many GW troops (Abdel-Rahman *et al.*, 2004; Sullivan *et al.*, 2018). In addition, certain populations of veterans may have been exposed to nerve gas agents such as sarin and cyclosarin (Haley *et al.*, 2022; RACGWI, 2014). To mitigate risk of fatal exposure to sarin, the prophylactic agent pyridostigmine bromide (PB) was commonly prescribed to troops, and this medication was often overused (Marino *et al.*, 1998). Collectively, these are known as the Gulf War Chemicals (GWC).

Epidemiological and Clinical Evidence

A growing body of evidence has linked GWC to the chronic GWI symptoms across both human veterans and preclinical animal models. This, combined with toxicological studies conducted on these chemicals, has highlighted the biological implications behind GWC exposure

during the Gulf War. Such studies have shown that veteran contact with these substances is the most probable etiological factor for GWI (Binns, 2008; Proctor *et al.*, 1998; Shine, 2014). However, the exact levels of chemicals to which the veterans were exposed is difficult to elucidate. This, along with the variety of chemicals, duration of exposure, and the diverse dosages that troops may have been exposed to has made studying the disease a complicated matter. As such, epidemiological evidence is considered through several approaches: exposure reports, deployment demographics, and the veterans themselves have been studied. Clinical evidence from studies utilizing Gulf War veterans have been compared to toxicological profiles to determine the connection between GWC exposure and reported symptomology. Collectively, epidemiology-based research has been instrumental for understanding Gulf War Illness and the effects on Gulf War veterans.

Thirty years have passed since the war, but Gulf War veterans continue to suffer from GWI with little to no improvement in symptoms. This is due to the complexity of the disease and lack of knowledge behind its underlying pathology. Veterans suffering from GWI often experience multisymptomatic issues affecting the nervous system, gastrointestinal system, joints, muscles, as well as dermatological aberrations (White *et al.*, 2016). The two most commonly reported categories of symptoms in veterans suffering from GWI are neurological problems as well as physical pain within the muscles and joint (Binns, 2008). Symptoms that impact these two systems can be broadly categorized into the central nervous system (CNS) effects and peripheral effects of GWI. It is thought that inflammation, initiated by GWC, results in an inflammatory shift within the immune system, which may be the driving factor behind this disease (Alshelh *et al.*, 2020; Broderick *et al.*, 2013; Carpenter *et al.*, 2020; Trageser *et al.*, 2020). GWI veterans have been shown to have higher level of circulating inflammatory cytokines, chemokines, and

acute phase proteins (Broderick *et al.*, 2013; Garza-Lombó *et al.*, 2021; Hodgin *et al.*, 2022). It is thought that this increase in widespread inflammation may drive the peripheral and neurological abnormalities in GWI.

Neurological symptoms are prevalent in veterans suffering from GWI. Studies have found significant cognitive decline in deployed GW veterans. Short term memory, attention, and response speed has been reported to be significantly decreased in veterans with GWI (Anger *et al.*, 1999; Storzbach *et al.*, 2001; Toomey *et al.*, 2009). Self-reported exposure to GWC has been correlated with poorer neurophysiological test results in GW veterans (White *et al.*, 2001). Investigations have linked some of these cognitive decreases to changes within the structure and functionality of the brain in GWI patients. Significant changes to the subcortical brain (brainstem, cerebellum, thalamus), reduced cortical volume, and total reduced gray and white matter volume have been confirmed by a number of studies (Chao *et al.*, 2011; Chao *et al.*, 2016; Christova *et al.*, 2017) comparing GWI patients compared to non-GWI veteran controls. Reduced hippocampal volumes have also been reported in GW veterans, particularly those who were exposed to nerve agents (Chao *et al.*, 2017). Collectively, this body of evidence highlights the physiological and subsequent cognitive changes in the troops that had been exposed to GWC during the Gulf War.

Experimental Evidence and Preclinical GWI Models

While human studies of GWI have greatly contributed to the understanding of the disease, they are limited in what can be evaluated. During the war, the troops did not systematically document their exposures, and data regarding such events is primarily obtained via recall or through modeling (Kerr, 2015). Additionally, different subsets of troops may have been exposed to varying chemicals and concentrations based on their deployment and personal

habits (Binns, 2008). As a result, it is difficult to ascertain the full etiology behind GWI with human studies alone, and thus, animal models are also heavily utilized. Animal models treated with a variety of GWC combinations are often employed in the GWI field (Abdel-Rahman *et al.*, 2004; Abou-Donia *et al.*, 1996; Carpenter *et al.*, 2021; Kang *et al.*, 2000). To recapitulate GWI symptomology, several *in vivo* rodent models that use GW-relevant exposure combinations have been established and validated (Bryant *et al.*, 2021; O'Callaghan *et al.*, 2015; Zakirova *et al.*, 2015). These animal models have both recapitulated the changes noted in human veterans and provided means for further understanding of the neurobiological effects of GWC. Two of the most commonly utilized models used in GWI research are the pyridostigmine bromide and permethrin (PB + PM) model (Zakirova *et al.*, 2015), and the pyridostigmine bromide, N,N-diethyl-meta-toluamide, corticosterone, and diisopropyl fluorophosphate (PB/DEET/CORT/DFP) model (O'Callaghan *et al.*, 2015).

PB + PM Model

The PB + PM model involves 10 days of exposure to the pesticide PM and the prophylactic agent PB. PM works by binding to and subsequently delaying the closing of voltage-gated sodium channels leading to an influx in neuronal activity (Ray & Fry, 2006). PB is a reversible acetylcholinesterase inhibitor, which was prescribed to prevent the detrimental, possibly lethal, effects of nerve agent exposure (Hernandez *et al.*, 2019). This exposure paradigm arises from the high number of veterans who reported utilizing permethrin-based sprays while taking PB pills (Binns, 2008; Hill *et al.*, 2008). The short duration of exposure time in this model is reflective of the brief period of ground combat experienced by the troops during the Gulf War. Rodent studies utilizing the PB + PM model have shown it to induce sensorimotor deficits, decreases in memory formation, and alteration in neurotransmitter levels markers (Abdel-

Rahman *et al.*, 2004; Carpenter *et al.*, 2020). In addition, this model has been associated with neuroinflammation and changes to the blood brain barrier, resulting in neuronal death, astrogliosis, and upregulation of neuroinflammatory factors (Carpenter *et al.*, 2020; Joshi *et al.*, 2019; Zakirova *et al.*, 2015).

PB/DEET/CORT/DFP Model

The PB/DEET/CORT/DFP model involves 14 days of exposure to PB/DEET. On day 7, CORT is administered via the drinking water until day 14, and then finally, on day 15, a single injection of DFP is given (O'Callaghan *et al.*, 2015). DEET is an insect repellent and a mild acetylcholinesterase inhibitor that is known to be neuroinflammatory in high doses (Schoenig *et al.*, 1993; Sudakin & Trevathan, 2003). CORT is a murine stress hormone that is used as a surrogate for the war theater stress experienced by troops during the war. Sub-chronic exposure to CORT has been associated with an increase in the neuroinflammatory response to certain neuroinflammatory agents (Kelly *et al.*, 2012). DFP is an organophosphate that serves as a sarin surrogate. Exposure to DFP results in a variety of detrimental neurological effects including cognitive deficits and inflammation (Terry *et al.*, 2011). This model has been shown to induce widespread neuroinflammation within the prefrontal cortex, hippocampus, and striatum (O'Callaghan *et al.*, 2015). The combination of PB/DEET/CORT/DFP results in an upregulation in inflammatory and microglial activation factors within the ventral hippocampus (Carpenter *et al.*, 2020). Consequently, exposure to these GWC causes changes to motor function, anxiety-like behaviors, and short-term memory (Carpenter *et al.*, 2022).

In Vitro Models

While *in vivo* GWI studies have been instrumental to understanding the disease-specific effects of individual GWC or combinations, cell-level effects cannot be measured readily

(Ribeiro & Deshpande, 2021). Indeed, the effect that GWC may have on specific cell types is more difficult to elucidate in *in vivo* studies. As such, the mechanisms and subsequent impact of potential GWC-induced dysregulation may not be fully investigated. *In vitro* studies offer the precise control of GWC administration and the ability to test multiple exposure paradigms. Few *in vitro* GWI studies are currently published, and very limited studies are currently available that analyze GWC exposure models and the effects on neuroimmune cells. *In vivo* studies have shown microglial and astrocyte activation in GWI (Carpenter *et al.*, 2020; Zakirova *et al.*, 2015). Recently microglia have been shown to be associated with an increase in GWI driven neuroinflammation (Alshelh *et al.*, 2020; Garza-Lombó *et al.*, 2021). However, *in vitro* GWI studies featuring microglia are notably underrepresented in the literature, with neuronal cultures being predominantly studied (Azzolin *et al.*, 2017; Georgopoulos *et al.*, 2018; Naughton *et al.*, 2021; Tsilibary *et al.*, 2020). Such studies have shown that neuronal cells treated with serum from GWI patients had changes to network variability and significantly increased rates of neural apoptosis, implying that GWI veterans have inflammatory factors in circulation that could be contributing to neuroinflammation (Georgopoulos *et al.*, 2018). *In vitro* studies, such as these, have highlighted the contribution of neuroimmune crosstalk in GWI-driven neuroinflammation.

Contribution of the Immune System in Neuroinflammation

Neurological symptoms are highly reported in GWI, with fatigue, headache, cognitive decreases, and sleep disturbances being common complaints among veterans. In addition, it has been noted that veterans have an increased risk of developing neurodegenerative disorders, such as multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's disease, and certain brain cancers (Binns, 2008; White *et al.*, 2016). Furthermore, GWI veterans have been shown to have alterations in the immune system, resulting in decreased integrity of the blood brain barrier

(Georgopoulos *et al.*, 2017). The neurological abnormalities noted in GWI have been proposed to be a result of neuroimmune dysregulation and subsequent inflammation (Broderick *et al.*, 2013; Parkitny *et al.*, 2015; Skowera *et al.*, 2004). These studies have underscored a connection between GWC evoking immune dysfunction, leading to these aforementioned deleterious effects.

Neuroinflammation has been cited as an important contributing factor in many CNS based diseases and it can be initiated in response to injury, toxicants, or excessive stress. Once initiated, the neuroinflammatory response is mediated by an increase in the production of pro-inflammatory cytokines, chemokines, and secondary messengers. This inflammatory response leads to a recruitment of immune cells, which, in cases of prolonged duration and intensity, can lead to tissue damage and neuronal death (DiSabato *et al.*, 2016). This mechanism of immune-mediated neuroinflammation has been documented as a prevalent driving factor behind several diseases (Becher *et al.*, 2017; Elenkov *et al.*, 2005; Meduri *et al.*, 2009). Alzheimer's disease (Bisht *et al.*, 2018; Katsumoto *et al.*, 2018; Montagne *et al.*, 2017), multiple sclerosis (Amato *et al.*, 2006; Chiaravalloti & DeLuca, 2008; Rodriguez Murua *et al.*, 2022), and Parkinson disease (Tan *et al.*, 2020; Tansey *et al.*, 2022) have all been characterized by disruptions in immune system function and subsequent neuroinflammation. Immune dysregulation has been shown to result in an increase in unregulated inflammation in both the periphery as well as within the CNS (Becher *et al.*, 2017). Further, aberrant communication between the CNS and periphery has been cited as a key factor leading to neuroimmune dysregulation and subsequent neuroinflammation (Stephenson *et al.*, 2018).

Role of Microglial Activation in Neuroinflammation

Microglia are the resident macrophages of the CNS and are associated with driving essential functions during disease as well as homeostasis (Karperien *et al.*, 2013). Microglia perform a

variety of both neuroinflammatory as well as neuroprotective supportive functions by adapting to micro-environmental changes (Aloisi, 2001). Microglia are often grouped into two states; resting and activated phenotypes. M0 or precursor microglia cells are associated with homeostasis, and they work to maintain a balanced physiological state (Zhang *et al.*, 2018). Once presented with stimuli, microglia can take on two broadly activated phenotypes: the M1-like pro-inflammatory phenotype or the M2-like anti-inflammatory phenotype. M1-like microglia are activated by LPS and IFN-γ, which results in activation of the NF-κB pathway to induce the production of several pro-inflammatory cytokines, such as IL-6 and TNF-α. Alternatively, M2-like microglia are primarily associated with regeneration and repair, and are prompted by cytokines such as IL-4, IL-13, and IL-10 (Tang & Le, 2016). Acutely, activated microglia help to resolve pathogenic and harmful events; however, excessive activation can lead to deleterious effects. Dysregulation of M1-like activated microglia can cause a hyperactivated state resulting in an increase in inflammatory cytokines, neuroinflammation, and subsequent neuronal damage (Block *et al.*, 2007). Hyperactivated microglia have been implicated as the catalyst for neuroinflammation in several neurodegenerative diseases (Bisht *et al.*, 2018).

Neuroinflammation in GWI

Neurological deficits are among the most highly reported symptom criteria in veterans suffering from GWI and are thought to result from an increase in neuroinflammation (RACGWI, 2014). Studies have linked neuroinflammation in GWI patients to an increase in depressive and anxiety-like behaviors, as well as a decrease in learning and memory abilities (Carpenter *et al.*, 2022; Parihar *et al.*, 2013; White *et al.*, 2016). Although GWI consists of a variety of neurological symptoms, it is not known whether neuroinflammation is initiated directly from within the CNS or indirectly from systemic inflammation in the periphery. Within the CNS,

microglial hyperactivation has recently been explored as a potential cause behind GWI neuroinflammation. Studies have linked hyperactive microglia to a dysregulation of functionality, resulting in an increase in pathogenic phagocytosis as well as pro-inflammatory cytokine production, ultimately leading to neuroinflammation (Bisht *et al.*, 2018; Katsumoto *et al.*, 2018; Madore *et al.*, 2020). This correlation between microglia and neuroinflammation in GWI was recently confirmed in an imaging study linking neuroinflammation in brain regions to an upregulation of microglial activation genes in GWI veteran brains. (Alshelh *et al.*, 2020).

Exposure to GWC may serve to increase the inflammatory response of neuroimmune cells, particularly in microglia. Within the brain, it has been shown that exposure to GWC causes an inflammatory shift within the immune system, leading to an overexpression of inflammatory mediators (Carpenter *et al.*, 2020; Georgopoulos *et al.*, 2018; O'Callaghan *et al.*, 2015; Zakirova *et al.*, 2015). GWC have been shown to increase pro-inflammatory cytokine production as well as upregulate the microglial activation marker F4/80 (Carpenter *et al.*, 2022; Carpenter *et al.*, 2020). DEET, PM, and PB have been associated with an increase in microglial inflammation within the rat hippocampus (Parihar *et al.*, 2013). Activated microglia have been implicated as a driving factor behind fibromyalgia, a disease that shares several symptoms of GWI, such as fatigue, chronic pain, and memory deficits (Alshelh *et al.*, 2020; Yasui *et al.*, 2014). In this regard, murine GWI studies have shown microglial activation and neuroinflammation in multiple GWI models (Carpenter *et al.*, 2020; O'Callaghan *et al.*, 2015; Parihar *et al.*, 2013). This led to the theory that GWI may be, in part, due to microglial dysregulation stemming from GWC exposure. This dysregulation could result in significantly upregulated production of pro-inflammatory factors as well as a change in the ability of these cells to perform other vital functions, such as phagocytosis – another key regulatory role of microglia. We hypothesized that

this dysregulation of microglial cells could be a driving factor behind the neuroinflammation effects observed in GW veterans suffering from GWI.

Immune cell dysregulation and systematic inflammation, within both the CNS and periphery, has been documented in GWI patients (Georgopoulos *et al.*, 2017; Parkitny *et al.*, 2015; Trageser *et al.*, 2020). The indirect effects of peripheral influence on the neuroinflammatory effects of GWI are not well understood. GWI has been shown to result in widespread dysregulation of several systems including the liver, spleen, and gut (Carpenter *et al.*, 2020; Liu *et al.*, 2021; Michalovicz *et al.*, 2019; Mote *et al.*, 2020). Inflammation from the periphery has been shown to indirectly impact the brain by both initiating and exacerbating neuroinflammation (Georgopoulos *et al.*, 2018). The increase in circulating inflammatory factors in GWI veterans has been linked to increasing microglial activation (Garza-Lombó *et al.*, 2021; Johnson *et al.*, 2016). While microglial activation and circulating inflammatory factors in GWI has been documented, the extent of this interaction remains unknown.

Significance of Thesis Research

In the context of neuroimmune functionality during GWI, there are very few published studies available. The lack of exploration of microglial involvement in the etiology of GWI prompted the present study to explore the potential link between GWC and microglial cells. Understanding the impact of GWC on neuroimmune cells could provide new insights into the disease, resulting in the potential exploration of new therapeutic avenues. This study aimed to broaden the understanding of GWI by evaluating the impacts of GWC on a cellular level, specifically in microglia. To do this, we evaluated the direct and indirect effects of GWC on microglial cells. While analyzing the indirect effects, we looked at peripheral and tissue response to GWC by using plasma and an *ex vivo* slicing technique.

Firstly, we wanted to evaluate the impact of GWC on microglia to determine if GWC exacerbates the microglial inflammatory response. To accomplish this, we performed a series of *in vitro* exposure experiments utilizing a variety of GWC combinations. After developing these models, we then explored the correlation between GWC and microglial activation by evaluating the production of pro-inflammatory cytokines. Cytokine production was analyzed in response to both treatment and pretreatment with GWC followed by a challenge with an inflammatory stimulus. To further categorize the neuroimmune response, we utilized an *ex vivo* model which will enable the tissue neuroimmune response to GWC to be investigated.

We also explored the interaction between peripherally induced inflammation and microglia. Studies have shown that GWI veterans have higher levels of circulating inflammatory biomarkers over aged-matched healthy veterans (Johnson *et al.*, 2016; Vashishtha *et al.*, 2020). Acute phase proteins (APPs) have been investigated recently as a potential diagnostic biomarker of GWI. APPs are nonspecific serum proteins produced by the liver that increase in conjunction with inflammation (Jain *et al.*, 2011). GWI veterans have been shown to have an increase in serum APPs, raising the question of whether APPs could be contributing to some of the etiology of GWI (Hodgin *et al.*, 2022; Johnson *et al.*, 2016). We analyzed GWI plasma from two of our lab's GWI studies to determine if APPs are affected by GWC exposure. We also utilized GWI plasma to investigate indirect peripheral effects of GWC on microglial cells. Comprehensively, this study provides insight as to how GWI neuroinflammation may induce or be exacerbated by microglia. In identifying the impact of GWC on these cells, we aimed to be able to identify possible approaches for remediating the neuroinflammatory impacts of GWI.

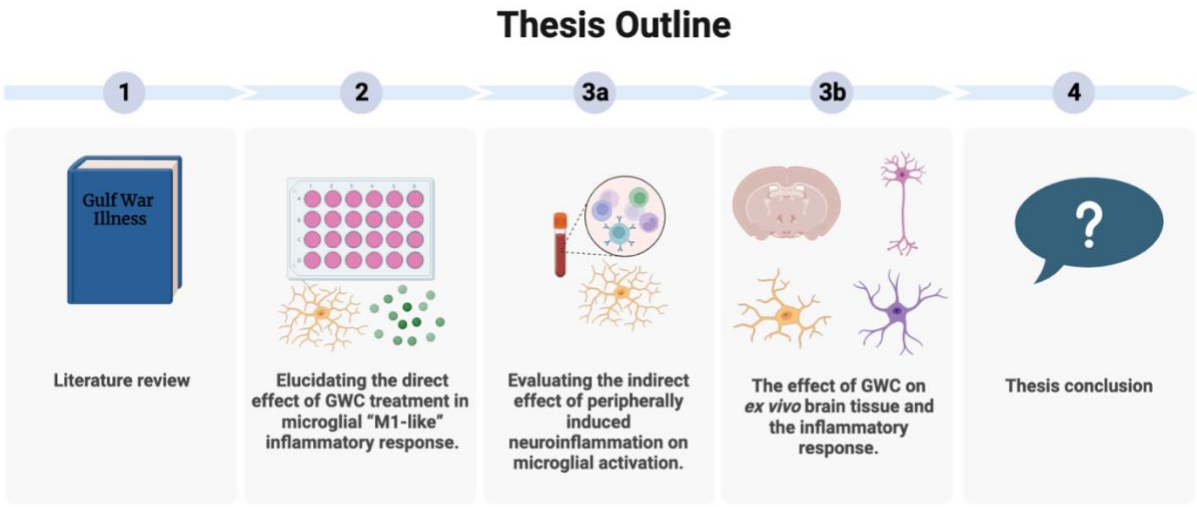


Figure 1.1: Summary of Thesis Direction

Chapter 2

Elucidating the direct effect of GWC treatment in microglial "M1-like" inflammatory response

Introduction

Neuroimmune dysfunction and subsequent chronic inflammation have been implicated as a critical factor in the etiology of many neurodegenerative diseases (Streit *et al.*, 2004). Immune-mediated neuroinflammation has been shown to result in neuronal damage, death, and subsequent decline in cognitive function (d'Avila *et al.*, 2018; Zhao *et al.*, 2019). Recently, microglia have been implicated with a role in generating and exacerbating inflammation within the central nervous system (CNS). Microglia are the resident innate immune cell of the brain, and they play an integral role in neuroprotection as well as neuroinflammation. Microglia perform the vital task for regulating the inflammatory response within the CNS. While inflammation is a vital defense implemented by the immune system to protect from disease, it can become detrimental when it becomes uncontrolled or chronic (Stephenson *et al.*, 2018). Microglia can contribute to chronic inflammation when they become hyperactivated in response to insult, toxicant exposure, or injury. Hyperactivated microglia have been shown to have significantly increased production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-a) and interleukin-6 (IL-6) (Alvarez *et al.*, 2007).

Neuroinflammation has been investigated as the culprit behind some of the symptoms noted in Gulf War Illness (GWI), a chronic disease afflicting close to 200,000 (approximately

one third) of the U.S. troops deployed during the 1990 – 1991 Persian Gulf War (White *et al.*, 2016). GWI is a multi-symptomatic disease that has a variety of repercussions including cognitive impairment, behavioral changes, gastrointestinal abnormalities, and joint and/or muscle pain (Kang *et al.*, 2000; Kimono, 2021; White *et al.*, 2001). The exact etiology behind GWI has not been elucidated, but it is thought to be a combination of exposure to hazardous materials present in the war, including pesticides and insect repellents, such as permethrin (PM) and N-N, Diethyl-3-methylbenzamide (DEET), which were used in excess by a large population of GW veterans. (Abdel-Rahman *et al.*, 2004; Sullivan *et al.*, 2018). Exposure to the nerve gas agents, sarin and cyclosarin, occurred in a subset of troops. To mitigate risk of fatal exposure to sarin, the prophylactic agent pyridostigmine bromide (PB) was commonly prescribed to soldiers at risk of nerve agent exposure (Marino *et al.*, 1998). Collectively, these are known as the Gulf War Chemicals (GWC). It is speculated that this combination of chemical exposure, along with the intense stress experienced during warfare may converge to produce the unique syndrome experienced by Gulf War veterans (Abdel-Rahman *et al.*, 2004; Kang *et al.*, 2000; White *et al.*, 2016).

It has been suggested that exposure to GWC might serve to exponentially increase the inflammatory response of the immune system, which results in the symptoms of GWI. Within the brain, it has been shown that exposure to GWC causes an inflammatory shift within the innate immune system, leading to an overexpression of inflammatory mediators (Garza-Lombó *et al.*, 2021; Parihar *et al.*, 2013; Skowera *et al.*, 2004). Microglia are the most prevalent innate immune cell in the CNS, and upregulation in the brain-wide neuroinflammatory response has been tied to microglial activation (Block *et al.*, 2007; Schramm & Waisman, 2022). GWC have been shown to increase pro-inflammatory cytokine production as well as upregulate microglial

activation marker F4/80 (Carpenter *et al.*, 2020). In animal studies, DEET, PM, and PB have been linked to an increase in microglial inflammation within the rat hippocampus (Parihar *et al.*, 2013). As such, we surmised that GWC exposure results in microglial hyperactivation, which could cause significantly upregulated production of the pro-inflammatory factors noted in GWI. Microglial hyperactivation could be a driving factor behind the neuroinflammatory effects observed in GWI veterans. The lack of exploration of microglial involvement in the etiology of GWI prompted the present study to explore the potential link between GWC exposure and increased microglial inflammatory response. We sought to emulate a series of *in vitro* exposure paradigms involving a variety GWC combinations to which veterans were subjected during the war. After developing these models, we wanted to explore the link between GWC and the microglial inflammatory response.

The objective of this chapter was to explore the direct effect of GWC on microglial activation. We theorized that GWC will cause microglial hyperactivation by causing an increase in pro-inflammatory cytokine production. Previous studies have shown a correlation between GWC and immune dysregulation (Georgopoulos *et al.*, 2017; Trageser *et al.*, 2020). GWC have been associated with changes in immune cell function, as well as increases in inflammatory cytokines, within both the CNS and in the peripheral circulation (Bryant *et al.*, 2021; Hodgin *et al.*, 2022). *In vitro* studies have linked the insecticide permethrin to an increase in microglial activation (Hossain *et al.*, 2017) while DEET has been shown to cause neurotoxicity in conjunction with certain chemicals (Schoenig *et al.*, 1993). The theory behind this aim is two-fold. First, GWC exposure could cause pro-inflammatory microglial responses on their own. Second, GWC may serve to significantly exacerbate the microglial M1-like inflammatory response by increasing pro-inflammatory cytokine production. In other words, this study seeks to

determine if GWC could be causing a direct increase in microglial-driven inflammatory cytokine production. If proven, this interaction may be a critical component in understanding neuroinflammation in patients suffering from GWI.

To investigate the effect of GWC on microglia, we utilized two *in vivo* GWI models (the O'Callaghan *et al.,* 2015 and Zakirova *et al.,* 2015 models) that were used the Carpenter *et al.,* 2020 and 2022 studies from our laboratory. In these studies, F4/80, IL-1β, and TNF-α were upregulated in the hippocampus of GWI mouse models, suggesting the potential role of microglia in moderating GWI-induced neuroinflammation (Carpenter *et al.,* 2021; Carpenter *et al.,* 2022; Carpenter *et al.,* 2020). We sought to use versions of these GWI models *in vitro* to establish if GWC will trigger microglia directly, and if so, what the mechanism is for GWI-driven microglial activation. To determine this, we exposed N9 microglial cells to pyridostigmine bromide (PB) and permethrin (PM) (as used in the Zakirova model), as well as a combination of PB, corticosterone (CORT), N-N, Diethyl-3-methylbenzamide (DEET), and diisopropyl-fluorophosphate (DFP) (as used in the O'Callaghan model). These models henceforth are referred to as the PB + PM model and the DFP model. Both models were used in conjunction with and in the absence of the inflammagen lipopolysaccharide (LPS) to evaluate the potential synergistic or additive impact of GWC. We hypothesized that GWC treatment will directly exacerbate the pro-inflammatory response of microglial cells, and that baseline inflammation, evoked by LPS exposure, will be dramatically increased by GWC exposure. GWC treatment consisted of physiologically relevant GWI models, based on the PB + PM and DFP models.

Methods and Materials

Gulf War Chemicals

N-N, Diethyl-3-methylbenzamide (DEET, Milliporesigma, St. Louis, MO), permethrin (PM; 28% cis/71% trans isomer; 99% purity; ChemServices Inc., West Chester, PA), pyridostigmine bromide (PB; ≥ 98% purity; Sigma Aldrich, St. Louis, MO), diisopropylfluorophosphate (DFP, Sigma Aldrich), and corticosterone (CORT; Steraloids, Newport, RI) were used in the study. DFP served as a sarin surrogate, while CORT was utilized to simulate war-theater stress.

Cell Culture

An N9 murine microglial line was utilized for this study. This cell line was derived from retroviral immortalization of embryonic mouse brain cultures collected on day 13 (Righi *et al.*, 1989). The culture was maintained (at 37 °C in 5% CO2, 95% air) in a RPMI complete medium formulated with RPMI medium 1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals now Bio-Techne, Minneapolis, MN), 1% sodium pyruvate 100 mM (Gibco), 1% non-essential amino acids (Fisher Scientific, Waltham, MA), 1% sodium bicarbonate (Gibco), 1% penicillin streptomycin (Gibco), 1% L-glutamine (Gibco), 0.1% Amphotericin B, and 0.1% 2-mercaptoethanol (Gibco).

Selection of Relevant GWC *In Vitro* Dosages

To select biologically relevant *in vitro* dosages of the DEET, PM, PB, DFP, and CORT, we analyzed the toxicology literature on each chemical. We evaluated several studies assessing the pharmacokinetics of each chemical after relevant administration methods. PB dose was based on serum concentration after oral administration (Marino *et al.*, 1998). DFP dosages are based on serum concentrations after SQ injection (Terry *et al.*, 2011). DEET is based of topical

exposure (most common application during the war), while CORT is based on mice that had experienced varying degrees of stress (Fediuk *et al.*, 2010; Gong *et al.*, 2015). PM is based on dosages used in prior studies by our lab (Carpenter *et al.*, 2020; Zakirova *et al.*, 2015). Several relevant concentrations of each chemical were selected to represent different exposure paradigms that veterans may have experienced during the Gulf War.

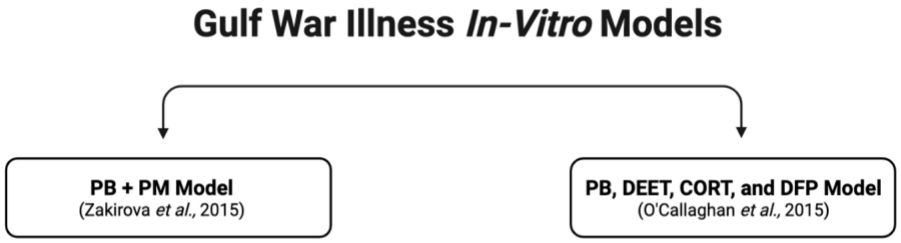


Figure 2.1. In Vitro GWI models used for this study.

In vitro GWC Exposure

Cells were seeded at 0.5×10^6 in a volume of 1 mL/well in a 24-well tissue culture safe plate. Cells were treated with the following GWI chemicals at varying concentrations noted below over several experiments. PM was utilized at 1, 10, and 20 µM. PB was used at doses of 5, 10, and 20 ng/mL. DFP was administered at 20, 200, and 1000 nM. DEET concentrations included 0.1, 1, and 10 µg/mL. CORT was utilized at 0.5, 1 and 1.5 µg/mL. Finally, lipopolysaccharide (LPS, Sigma) was used as an inflammagen at 1 ng/mL.

***In vitro* Pretreatment Experiments:**

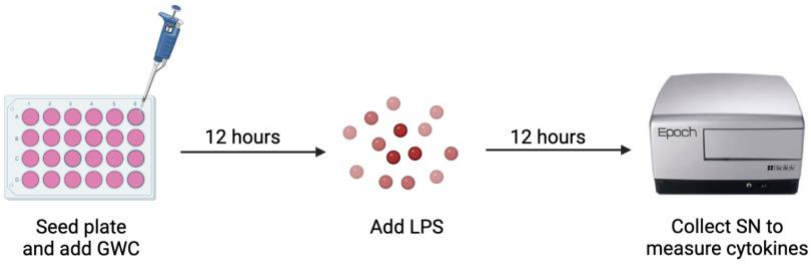


Figure 2.2. Summary of experimental design for the first subset of *in vitro* experiments

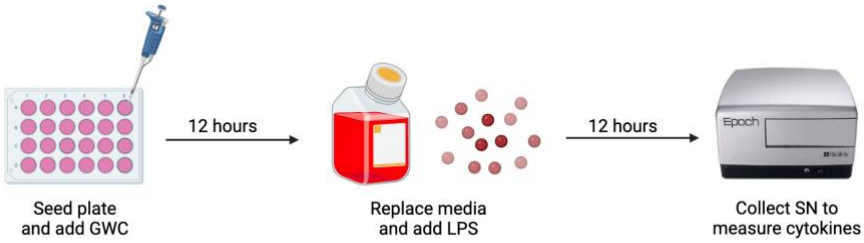


Figure 2.3. Summary of experimental design for the second subset of *in vitro* experiments

Cells were seeded at 0.5×10^6 cells/well onto 24-well tissue culture safe plates and exposed to various GWC (described in each figure caption in the results section). Our focus was to explore the potential for GWC microglial priming. After 12 hours, LPS was added, and exposure continued for 12 hours before the supernatant was collected and frozen at -20°C for cytokine analysis.

***In Vivo* to *In Vitro* Model Experiments:**

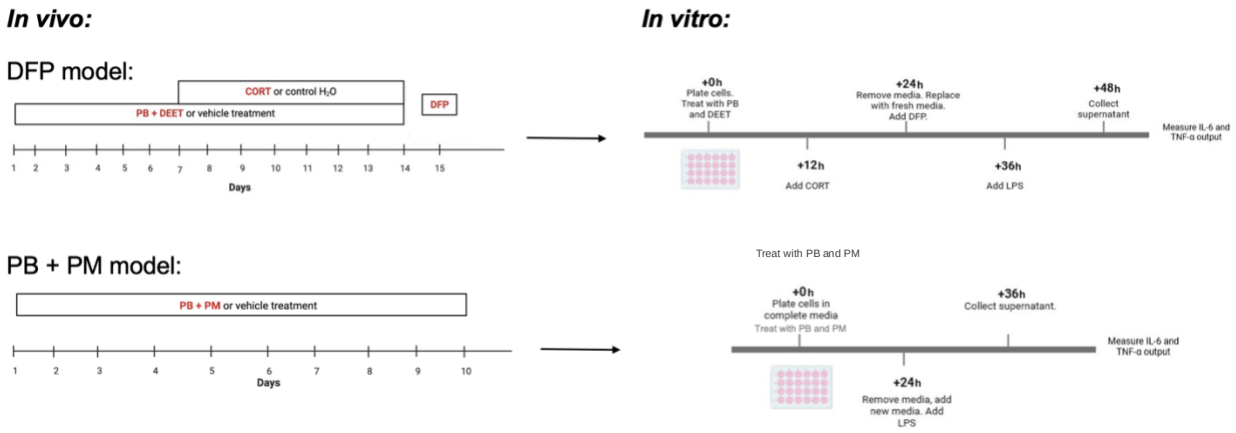


Figure 2.4. Summary of experimental design for the third *in vitro* experiments which used a *in vivo* to *in vitro* extrapolated model.

After the initial experiments, we sought to emulate more closely what was done *in vivo* by our lab. To accomplish this, we utilized two different methods for the PB + PM and DFP models. For all experiments the N9 cells were seeded at 0.5×10^6 cells/well onto 24-well tissue culture safe plates. For the DFP model, cells were plated and immediately treated with PB and DEET. After 12 hours, CORT was added. After an additional 12 hours, the media was removed and replaced with fresh RPMI complete media. Cells were then treated with DFP. Twelve hours after DFP treatment, cells were then challenged with LPS. After 12 hours of LPS exposure, the plate was spun down, and the supernatant was collected and frozen at -20°C for cytokine analysis.

For the PB + PM model, cells were plated in complete media and immediately treated with PB + PM. After 12 hours, the supernatant was removed, fresh RPMI supernatant was added,

and cells were treated with LPS. After 12 hours of exposure to LPS, the supernatant was collected and frozen for cytokine analysis.

Cytokine Analysis

To evaluate the levels of cytokines in the supernatant, ELISA was performed using Duo-Set ELISA kits (Bio-Techne, Minneapolis, MN). At the end of the exposure time, the plate was spun down, and the supernatant was removed for ELISA. Samples were run in duplicate following manufacturer's protocol. IL-6 and TNF-α were the two pro-inflammatory cytokines evaluated in this study.

Results

2.1. *In vitro* GWC Pretreatment Experiments

2.1.1. Cytokine analysis for the PB + PM model:

Exposure to the PB + PM model without removal before LPS challenge led to a significant decrease in LPS induced TNF-α secretion ($p \leq 0.05$, Figure 2.5). After this experiment, we changed the protocol to remove GWC pretreatment before the LPS challenge to more closely emulate the exposure scenarios experienced by GW veterans. Pretreatment and then removal of the extrapolated PB + PM model prior to LPS caused a significant increase at the highest dose selected, PB 20 ng/mL + PM 20 μM ($p \leq 0.001$, Figure 2.10).

2.1.2. Cytokine analysis for the DFP model:

First each component of the DFP model was analyzed on its own at several doses to determine the effect of each chemical on N9 microglial response by analyzing TNF-α and IL-6

secretion. These GWC were removed prior to the LPS challenge. DEET caused a significant decrease in LPS induced TNF- α at the highest dose, DEET 10 μg/mL ($p \leq 0.001$, Figure 2.6) and significant decreases in LPS induced IL-6 at all doses ($p \leq 0.05$, Figure 2.6). CORT decreased LPS induced TNF- α and IL-6 secretion at all doses ($p \leq 0.001$, Figure 2.7). DFP decreased LPS induced TNF- α secretion at 200 nM ($p \leq 0.05$, Figure 2.8). A combination of both CORT at 1 μg/mL and DFP at 200 and 1000 nM resulted in a decrease in LPS induced TNF- α secretion ($p \leq 0.001$, Figure 2.9). Likely due to the anti-inflammatory effect of CORT. The complete DFP model resulted in a decrease in LPS induced TNF- α secretion ($p \leq 0.001$, Figure 2.11), highlighting the acute anti-inflammatory effect of this model on N9 microglial inflammatory response.

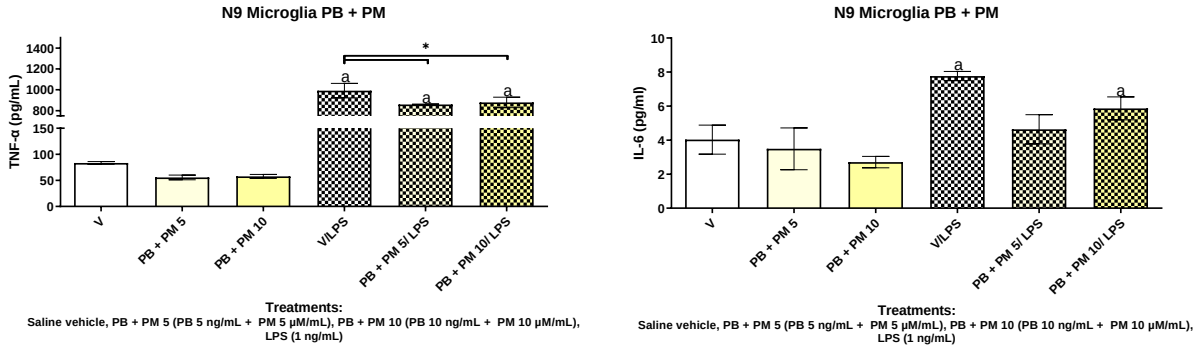


Figure 2.5. The effect of PB or PM on TNF-α and IL-6 production in N9 microglia.

N9 microglial cells were seeded at 0.5×10^6 onto a 24-well plate. After plating, cells were primed with a combination of PB (10 ng/mL or 5 ng/mL) and PM (10 μM or 5 μM). After 12 hours of priming, cells were treated with a saline vehicle or LPS (1 ng/mL). Supernatant was collected 12 hours after the saline or LPS treatment, and TNF-α and IL-6 ELISAs were performed. On their own, PB and PM did not change TNF-α or IL-6 secretion. In conjunction with 1 ng/mL of LPS, PB and PM caused a slight decrease in both cytokines. Neither PB nor PM

caused a significant increase in cytokines under any condition. Data is presented as mean ± SEM. GWC effects are indicated by * = $p \leq 0.05$. 'a' indicates a significant LPS effect at $p \leq 0.05$.

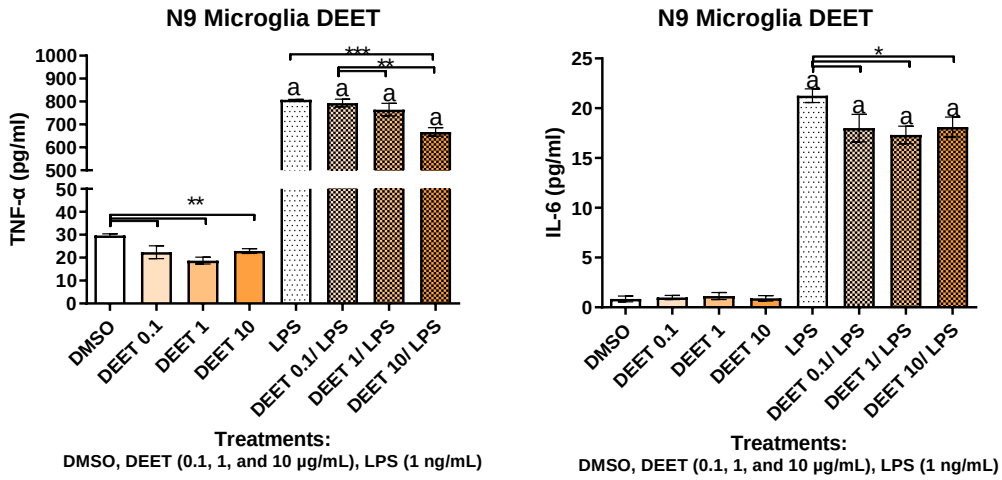


Figure 2.6. The effect of DEET priming on N9 microglial cytokine production.

In this experimentation we switched the timing of exposure to match more closely what has been done *in vivo* studies. The subsequent studies involved removing the GWC prior to the addition of LPS. N9 microglial cells were seeded at 0.5×10^6 onto a 24-well plate. After plating, DEET was administered at doses of 0.1, 1, or 10 µg/mL. After 12 hours, the media was removed and replaced with fresh medium with either vehicle or LPS (1 ng/mL) added. The supernatant was collected 24 hours after DEET application, and TNF-α and IL-6 ELISAS were performed. DEET alone did not cause a significant effect in inflammatory cytokine production. When given before LPS, DEET resulted in a significant decrease in both TNF-α and IL-6. Data is presented as mean ± SEM. GWC effects are indicated by *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$. 'a' indicates a significant LPS effect at $p \leq 0.05$.

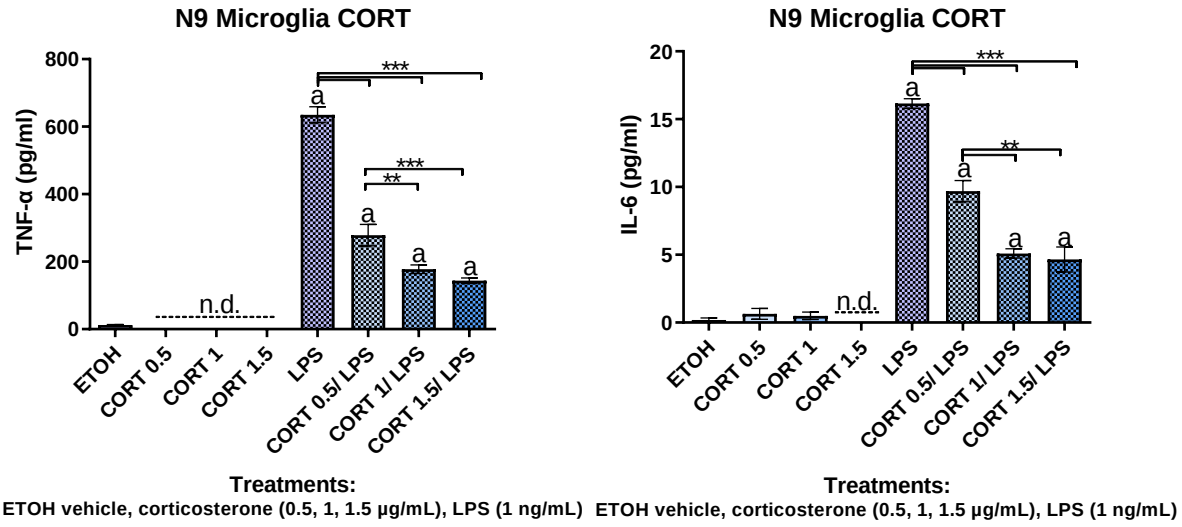


Figure 2.7. The effect of CORT priming on N9 microglial cytokine production.

N9 microglial cells were seeded at 0.5×10^6 onto a 24-well plate. After plating, a physiologically or super physiologically relevant dose of CORT (0.5, 1, and 1.5 µg/mL) was administered. After 12 hours the media was removed and replaced with fresh medium with either vehicle or LPS (1 ng/mL) added. The supernatant was collected 24 hours after CORT application, and TNF-α and IL-6 ELISAS were performed. CORT alone did not cause a significant effect in cytokine production. When given before LPS, CORT caused a dose dependent decrease in both TNF-α and IL-6 production. Data is presented as mean ± SEM. GWC effects are indicated by *** = $p \leq 0.001$, ** = $p \leq 0.01$. 'a' indicates a significant LPS effect at $p \leq 0.05$.

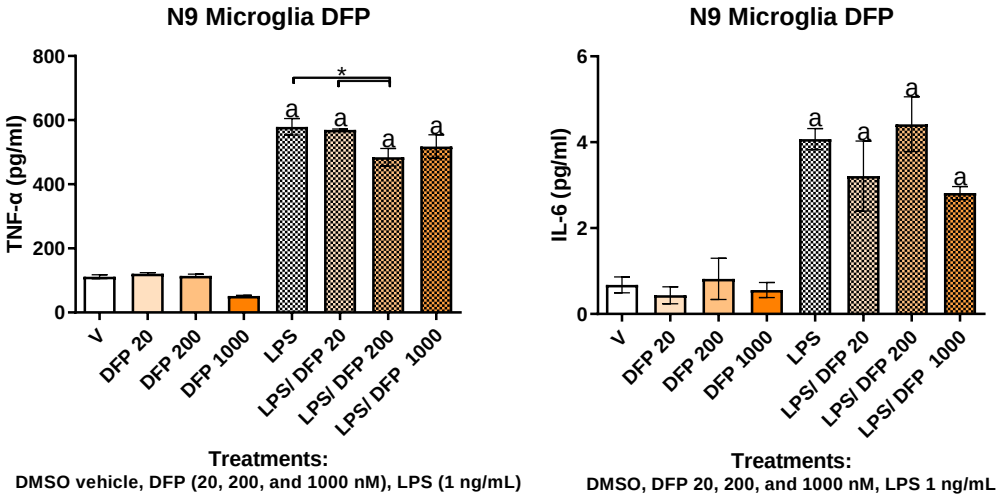


Figure 2.8. The effect of DFP priming on N9 microglia cytokine production.

N9 microglial cells were seeded at 0.5×10^6 onto a 24-well plate. Immediately after plating, DFP was administered at a physiologically low, moderate, and high dose (20, 200, and 1000 nM) to N9 microglia. After 12 hours, media was removed and replaced with fresh medium with either vehicle or LPS (1 ng/mL) added. Supernatant was collected 24 hours after DFP application, and TNF-α and IL-6 ELISAS were performed. DFP alone did not case a significant change in either cytokine. When given before LPS, DFP caused no significant change in either TNF-α and IL-6 production. Data is presented as mean ± SEM. GWC effects are indicated by * = $p \leq 0.05$. 'a' indicates a significant LPS effect at $p \leq 0.05$.

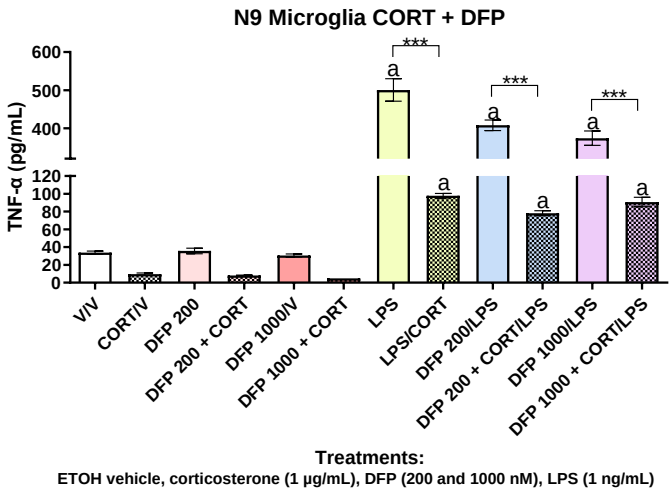


Figure 2.9. The effect of CORT and DFP priming on N9 microglial cells.

N9 microglial cells were seeded at 0.5×10^6 onto a 24-well plate. CORT (1 µg/mL) and DFP (200 or 1000 nM) were then administered separately and in conjunction to N9 microglia cells. After 12 hours, media was removed and replaced with fresh medium with either vehicle or LPS (1 ng/mL) added. The supernatant was collected 24 hours after CORT and DFP application, and TNF-α and IL-6 ELISAS were performed. Neither CORT nor DEET alone caused a significant change in either cytokine. When given together, CORT and DEET had a non-significant decrease in both IL-6 and TNF-α production. When given before LPS, CORT caused a significant decrease in both cytokines. DFP caused a trending decrease compared to LPS given by itself. A combination of DFP and CORT caused the most significant decrease in both cytokines at both dosages. Data is presented as mean ± SEM. GWC effects are indicated by *** = $p \leq 0.001$. 'ª' indicates a significant LPS effect at $p \leq 0.05$.

2.2 *In Vivo* to *In Vitro* Extrapolation Experiments:

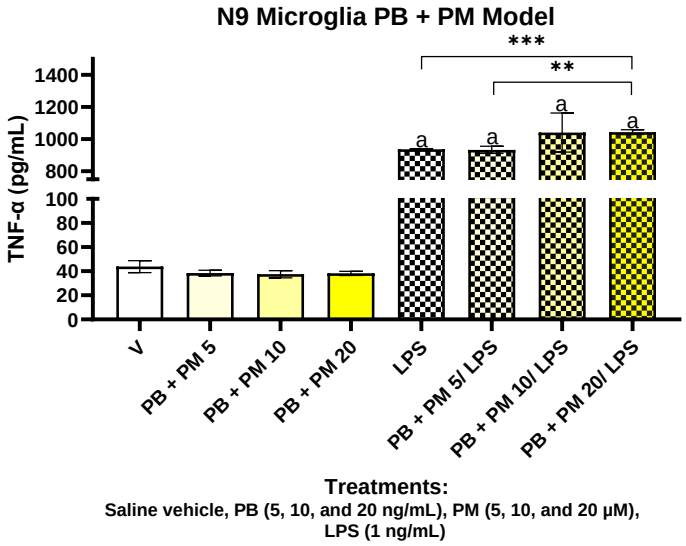


Figure 2.10. *In vivo* to *in vitro* extrapolated PB + PM model effect on TNF-α production in N9 microglial cells.

N9 microglial cells were seeded at 0.5×10^6 onto a 24-well plate and immediately treated with PB (5, 10, or 20 ng/mL) and PM (5, 10, or 20 µM). After 12 hours the supernatant was removed, fresh medium was added, and cells were treated with LPS (1 ng/mL). After 12 hours of exposure to LPS, the supernatant was collected, and TNF-α analysis was conducted via ELISA. No combination of PB + PM alone caused significant effect on TNF-α secretion. When combined with LPS, PB + PM 20 (PB 20 ng/mL with PM 20 µM) caused a significant increase in TNF-α production over the LPS control. Data is presented as mean ± SEM. GWC effects are indicated by *** = $p \leq 0.001$, ** = $p \leq 0.01$. 'a' indicates a significant LPS effect at $p \leq 0.05$.

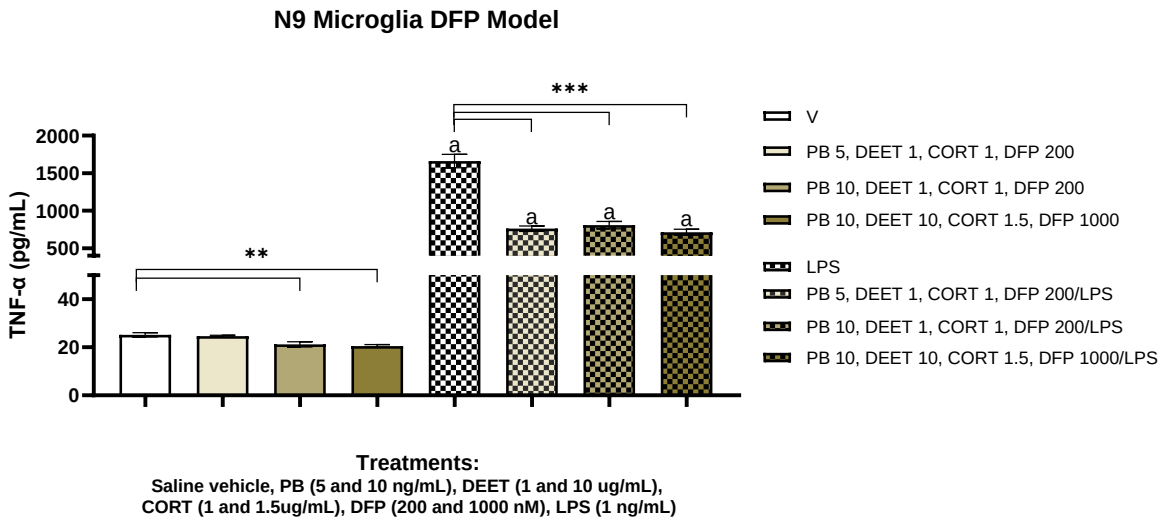


Figure 2.11. *In vivo* to *in vitro* extrapolated DFP model effect on TNF-α production in N9 microglial cells.

N9 microglial cells were seeded at 0.5×10^6 onto a 24-well plate and immediately treated with PB (5 or 10 ng/mL) and DEET (1 or 10 µg/mL). After 12 hours, CORT (1 or 1.5 µg/mL) was added. After an additional 12 hours, the media was removed and replaced with fresh media. Cells were then treated with DFP (200 or 1000 nM). Twelve hours after DFP treatment, media was again replaced, and the cells were then challenged with LPS (1 ng/mL). After 12 hours of LPS exposure, the plate was spun down, and the supernatant was collected for TNF-α analysis. No combination of PB, DEET, CORT and DFP alone caused a significant change in TNF-α secretion. When given with LPS, each dosage of the DFP model caused a significant decrease in TNF-α production from the LPS control. Data is presented as mean ± SEM. GWC effects are indicated by *** = $p \leq 0.001$, ** = $p \leq 0.01$. 'a' indicates a significant LPS effect at $p \leq 0.05$.

Discussion

With GWI impacting one-third of the veterans who served in the Gulf War and a lack of viable treatment options, it has become vital to understand the etiology of this disease to better target new therapeutic avenues (RACGWI, 2014). Neurological symptoms indicative of both structural and functional abnormalities within the brain have been observed in veterans suffering from GWI (Calley *et al.*, 2010; Christova *et al.*, 2017; Heaton *et al.*, 2007; Rayhan *et al.*, 2013). It is suspected that collective overexposure to GWC, including pyridostigmine bromide, pesticides such as permethrin and DEET, nerve agents, and war theater stress may play a causative role in GWI (Abou-Donia *et al.*, 1996; Kang *et al.*, 2000). While the exact etiology has yet to be elucidated, the innate immune system has been shown to be significantly disrupted in patients with GWI, and this may be a contributing aspect of the disease (Georgopoulos *et al.*, 2017; Johnson *et al.*, 2016) Microglia, one of the most numerous innate immune cells within the CNS, have been explored in recent years as a potential key causative factor in GWI (Parihar *et al.*, 2013). Microglia have been previously implicated in multiple neuroimmune diseases as major inflammatory contributors as a result of increased activation and subsequent release of pro-inflammatory cytokines (Aloisi, 2001). In this study, we investigated the response of microglia directly exposed to GWC to determine if these chemicals elicit an increased inflammatory change in these cells.

To determine the impact of GWC on microglia, we analyzed the pro-inflammatory cytokine response in microglial cells treated with GWC. It is noted that GW veterans experienced a variety of different chemical exposures during their deployment. To best encompass the myriad of exposure scenarios that may have been encountered by veterans, we utilized different combinations of GWC based on pharmacokinetic studies of each GWC. This

study focused on two of the most common established models used in GWI research, the PB + PM model and the DFP model. The PB + PM model has been shown to produce neurobehavioral and neuropathological consequences in animal models that are similar to the symptoms noted in veterans suffering from GWI (Joshi *et al.*, 2019; Sullivan *et al.*, 2018; Zakirova *et al.*, 2015). PM was a commonly used pesticide by GW troops, and blister packs of PB were distributed as a prophylactic medication to offer protection against nerve agent exposure. Our study implemented several concentrations of PB + PM to account for the variety of exposure paradigms that occurred during the war. We sought to explore the direct impact of these chemicals on microglia alone and with a subsequent inflammatory challenge to determine if GWC exposure results in an increase in microglial activation. We analyzed two methods of exposure for the PB + PM model, one in which PB + PM remained for the duration of the experiment, and one in which PB + PM were removed prior to LPS stimulation. In the absence of LPS, the PB + PM model does not cause a change in microglial inflammatory cytokine release. When PB + PM were present when LPS was administered, no potentiation in either cytokine occurred, and a decrease in TNF-α was noted. Whereas when PB + PM was removed prior to LPS stimulation, a potentiating effect caused by the higher doses of PB + PM occurred with TNF-α. Taken together, data from this model shows that when PB + PM are removed prior to stimulation with an inflammagen, an increase in inflammatory activation in microglial cells occurs. This same effect does not occur when PB + PM are still present.

The difference in microglial response to PB + PM may be due to the nature of these compounds. When taken acutely, PB has been shown to have an immunosuppressive effect in macrophages (Hernandez *et al.*, 2019). This may have resulted in the decrease in microglial inflammation noted in the cells that did not have PB + PM removed prior to LPS administration.

On the other hand, PM exposure has been linked to microglial activation and subsequent increase in inflammatory cytokine production (Hossain *et al.*, 2017). PM toxicity results from delaying the closing of the voltage-gated sodium channels (VGSC), resulting in a rapid influx in intracellular sodium that could result in microglial activation. It is possible that the inflammatory effect of PM is downregulated in the active presence of PB, and that continued presence of PB quells the inflammatory response in microglia. When PB + PM are removed before an inflammatory challenge, the immunosuppressive effect is not observed; leading to the possibility that brief exposure to, then discontinuation of exposure to the PB + PM model, may result in microglial priming. Since veterans began to experience a worsening of symptoms after exposure to GWC concluded after the war, it is possible that this priming effect represents part of the pathogenesis of the disease (Porter *et al.*, 2020). The effects of PB + PM may persist long after exposure ceases, leading to an increase in microglial susceptibility to a hyperactivated inflammatory response. After this point, we only utilized exposures to GWC that involved removing the GWC prior to LPS stimulation, as this most accurately reflects the *in vivo* models as well as veteran experiences.

The second model was the DFP model, which focused on exposure to PB, DEET, CORT, and DFP. These chemicals were analyzed for their effect on microglial cells alone and together as a model. This model has been used by our lab and others and has been shown to cause an increase in widespread neuroinflammation (Carpenter *et al.*, 2020; O'Callaghan *et al.*, 2015). The first chemical we analyzed individually was DEET. Several DEET dosages were selected based on the literature, which analyzed the effects of relevant GWI exposure levels and resulting blood plasma concentrations (Fediuk *et al.*, 2010). When DEET was applied to microglial cells, our results showed no significant increase in pro-inflammatory cytokine production in the presence

of increasing doses of DEET. Furthermore, DEET did not have a synergistic effect when combined with LPS. DEET, followed by LPS stimulation, resulted in a marked decreased in TNF-α production at the highest concentration of DEET used and significant decreases in IL-6 at all doses. There is a lack of evidence linking low doses of DEET to neurotoxic effects (Schoenig *et al.*, 1993). Although during the war, DEET was administered in high doses that are no longer used by the U.S. military (RACGWI, 2014), our study shows that even the higher doses of DEET do not trigger hyperactivation in microglia *in vitro*.

DFP was used as a surrogate for sarin exposure. DFP has been shown to cause a strong neuroinflammatory response in multiple brain regions (Carpenter *et al.*, 2020; Terry *et al.*, 2011). DFP doses were selected based on studies that analyzed plasma concentrations after several doses of DFP injection (Terry *et al.*, 2011). We did not see an inflammatory microglial response to DFP when it was given alone, with CORT, or when followed by LPS. CORT, a surrogate for war theater stress, has been known to enhance the neuroinflammatory effects of DFP *in vivo* when administered to rodents with the drinking water; however, we did not observe this effect in N9 microglia (O'Callaghan *et al.*, 2015). We selected biologically relevant concentrations of CORT based on studies that analyzed corticosterone levels in the blood plasma in response to increasing amounts of stress in mice (Gong *et al.*, 2015). When CORT was applied to microglia our results showed that CORT alone did not cause any change in microglial TNF-α and IL-6 production. When CORT treatment was removed prior to LPS stimulation, microglia responded to CORT exposure with a dose-dependent decrease in pro-inflammatory cytokine levels. Increasing the CORT dosage caused a decrease in both the TNF-α and IL-6 response to LPS. The anti-inflammatory effect of CORT persisted when it was given with DFP and both GWC were removed prior to LPS stimulation. After determining the effects of each chemical of the DFP

model on its own, we then combined these GWC to test the effect of the entire DFP model on microglia. The DFP model given in the absence of LPS did not cause any significant changes in microglial activation. When the DFP model was administered and removed prior to LPS stimulation, a significant decrease in inflammatory responses could be noted. The CORT within the DFP model drove decreases in TNF-α production in the presence of LPS. These data agree with *in vivo* studies which have demonstrated that the DFP model does not increase levels of M1-like microglia (Carpenter *et al.*, 2022; Carpenter *et al.*, 2020; O'Callaghan *et al.*, 2015).

When taken as a whole, these data suggest that the PB + PM model and the DFP model have opposite direct effects on microglial activation. No direct cytokine stimulatory effect was noted in either model in the absence of additional inflammatory stimulation. However, when LPS was administered after GWC pretreatment, it was noted that the PB + PM model had a stimulatory effect, while the DFP model had a suppressive effect. These data indicate that only the PB + PM model leads to microglial priming and only when these chemicals are removed prior to LPS stimulation. Microglial activation, as well as increases in pro-inflammatory cytokines have been noted in rodents treated with PB and PM (Abdullah *et al.*, 2016; Carpenter *et al.*, 2020; Parihar *et al.*, 2013; Saha *et al.*, 2021). Imaging studies have indicated that GWI veterans have increased levels of translocator protein (TSPO), a marker of microglial activation and neuroinflammation (Alshelh *et al.*, 2020). This study investigated whether GWC directly increase microglial priming leading to hyperactivation. Microglial priming causes an activated state in which microglia have an exaggerated response to a stimulus, which can lead to neuroinflammatory effects (Perry & Holmes, 2014). Studies have suggested that PB + PM can result in aberrations to microglial function and increases in inflammation (Carpenter *et al.*, 2020; Zakirova *et al.*, 2016; Zakirova *et al.*, 2015). Our data shows that the PB + PM model causes

microglial priming, which leads to an increase in inflammation. This microglial priming could be contributing to the neuroinflammatory changes noted in GWI veterans. These data suggest that veterans that were exposed to PB + PM during the GW may experience neuroinflammation as a result of microglial priming.

While the PB + PM model was shown to be immunostimulatory to microglia, the DFP model resulted in a decreased response to an inflammatory stimulus. The DFP model caused a significant decrease in LPS-mediated inflammation in microglial cells. These data suggests that the DFP does not contribute to microglial priming; instead, it leads to anti-inflammatory effects in stimulated microglia. This may be due to the presence of CORT, which can downregulate inflammation when given acutely (Liu *et al.*, 2018). Indeed, our study proved that CORT causes a strong immunosuppressive effect in microglia. DFP, as an irreversible AchE inhibitor may also result in an anti-inflammatory effect by increasing cholinergic signaling (Pavlov *et al.*, 2003). These data agree with the literature, which has shown that acutely (6 h) the DFP model results in a minor, primarily hippocampal microglial activation across multiple brain regions despite an overall increase of brain-wide neuroinflammation (O'Callaghan *et al.*, 2015). Other studies have associated this model with an upregulation in microglial markers F4/80 and YM-1, both of which can indicate the anti-inflammatory M2-like phenotype (Carpenter *et al.*, 2020; Greter *et al.*, 2015). Taken together, this study shows that the DFP model does not cause direct microglial inflammation and may instead cause M2-like activation. Indeed, this model has a different mechanism of initiating neuroinflammation that may originate from a different cell type or even from outside of the CNS. Our lab has previously shown the DFP model to result in astrogliosis and a decrease in neurogenesis implying that astrocytic activation and neuronal decrease may play a significant role in GWI pathogenesis (Carpenter *et al.*, 2022). Sarin exposure has been

associated with neuronal activation in the initial absence of microglia and astrocyte involvement (Angoa-Perez *et al.*, 2010). Thus, it may be possible that the DFP model does not induce acute microglial activation. Conducting these experiments with an astrocyte and/or neuron cell line would be helpful to determine the extent of the acute cellular effect of the DFP model. Overall, our findings help to further characterize the pathology behind GWI. Understanding the cellular contributions to GWI could help to identify new therapeutic targets for GWI patients, which would work to improve the quality of life for these veterans.

Chapter 3:

GWC effects on both peripherally induced microglial activation and regional *ex vivo* brain tissue inflammatory response

3a. Evaluating the indirect effect of peripherally induced neuroinflammation on microglial activation

Introduction

Gulf War Illness (GWI) is a chronic multisymptomatic disease affecting one third of the veterans from the 1990 – 1991 Persian Gulf War. Veterans with GWI can suffer from a constellation of symptoms including neurological, gastrointestinal, dermal, respiratory, and musculoskeletal abnormalities (White *et al.*, 2016). Chemical exposures to a variety of hazardous agents in the war theater, such as pesticides (permethrin and N, N-Diethyl-methylbenzamide), nerve agents (sarin and cyclosarin), nerve agent prophylactic medications (pyridostigmine bromide), along with stress or deployment and war, have been studied as the causative agents of GWI. Collectively, these Gulf War chemicals (GWC) have been shown to replicate similar symptoms in animal studies as have been noted in veterans suffering from GWI (Kerr, 2015; Ribeiro & Deshpande, 2021).

At present, the etiology behind GWI and the influence of GWC in the pathogenesis of this disease are still undetermined. Because GWI is a complex and multifaceted disease, it is possible that multiple mechanisms may be at work. Many of the symptoms of GWI are indicative

of both central nervous system (CNS) and peripheral immune dysfunction (Broderick *et al.*, 2013; Bryant *et al.*, 2021; Skowera *et al.*, 2004). Innate immune system dysregulation and inflammation have both been correlated with GWI (Johnson *et al.*, 2016). Veterans with GWI have been shown to have an increase in neuroinflammation and a disruption in the normal functionality of the blood-brain barrier (BBB), which may contribute to the neurological symptoms noted in the disease (Georgopoulos *et al.*, 2017; Joshi *et al.*, 2019). Indeed, studies have shown a significant upregulation in neuroinflammatory markers within GWI veterans prompting GWI to be studied as a neuroimmune disease (Alshelh *et al.*, 2020).

Neuroinflammation is caused by the persistent, unregulated activation of immune cells within the CNS (McGeer & McGeer, 2002; Streit *et al.*, 2004). However, neuroinflammation can be initiated from outside the CNS. Recently, aberrant communication between the CNS and the periphery has been implicated as a catalyst for neuroinflammation (Banks, 2015; Bechmann, 2005). Studies have shown that in some neurodegenerative diseases, peripheral immune dysregulation can significantly exacerbate disease pathogenesis (Montagne *et al.*, 2017). In addition, high levels of stress, as was experienced by troops during the GW, can cause a disruption in the BBB, leaving the CNS more vulnerable to the effects of peripheral inflammation (Banks, 2015). Indeed, stress has also been shown to cause microglial priming, resulting in increased expression of pro-inflammatory mediators (Schramm & Waisman, 2022). Microglia are the primary innate immune cell of the CNS and have been implicated in the pathogenesis of other neuroimmune diseases (Block *et al.*, 2007; Tang & Le, 2016). Microglia have been shown to contribute to the neuroinflammatory effects seen in GWI, but the exact mechanisms of GWC-driven microglial activation are not known (Alshelh *et al.*, 2020; Garza-Lombó *et al.*, 2021).

This study sought to determine whether GWC exposure results in an increase in peripheral inflammation. In addition, the study evaluated if circulating peripheral inflammatory factors can contribute to microglial hyperactivation in GWI. Systematically, the degree of severity of symptomology in GWI has been correlated to an increase in acute phase proteins, as well as other inflammatory proteins such as HMGB1, which a key player and a danger signal in multiple types (injury, infection, or sterile) of inflammation (Garza-Lombó *et al.*, 2021; Hodgin *et al.*, 2022; Vande Walle *et al.*, 2011). Furthermore, significant increases in pro-inflammatory cytokines, such as IL-6 and IL-1β, have been found in the serum of GWI patients (Butterick *et al.*, 2019). The indirect effects of circulating inflammatory GWI factors on microglial cells is something that has not yet been explored. Understanding this connection could be paramount to elucidating GWI neuroinflammatory etiology.

This study examined the effect of peripheral inflammation on the CNS immune response as well as to further understand the etiology of GWI neuroinflammation. To accomplish the peripheral-CNS component of this aim, the present study sought to analyze GWI plasma for circulating inflammatory proteins as well as to gauge the effect of these peripheral factors on microglial activation. Plasma from two, widely studied, murine, GWI models that our laboratory has investigated in prior experiments was analyzed. Acute phase proteins (APP) have been shown to be elevated in GWI patients (Hodgin *et al.*, 2022). APP have been used as a diagnostic measure in some chronic inflammatory diseases and may help to provide further information about the effects of GWI within the periphery (Jain *et al.*, 2011). Levels of circulating APPs within the plasma from both GWI models were assessed. In addition, experiments were conducted to evaluate the effects of peripheral factors present in GWI plasma on microglial activation to determine if they contribute to microglial hyperactivation.

Materials and Methods

Gulf War Chemicals

N-N, Diethyl-3-methylbenzamide (DEET, Milliporesigma, St. Louis, MO), permethrin (PM; 28% cis/71% trans isomer; 99% purity; Chem Services Inc., West Chester, PA), pyridostigmine bromide (PB; ≥ 98% purity; Sigma Aldrich, St. Louis, MO), diisopropylfluorophosphate (DFP, Sigma Aldrich), and corticosterone (CORT; Steraloids, Newport, RI). DFP served as a sarin surrogate, while CORT was utilized to simulate war-theater stress.

Cell culture

A N9 murine microglial line was utilized for this study. This cell line was derived from retroviral immortalization of embryonic mouse brain cultures collected on day 13 (Righi *et al.*, 1989). The culture was maintained (at 37 °C in 5% CO2, 95% air) in a RPMI complete medium formulated with RPMI medium 1640 (Gibco, Grand Island, NY) and supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals now Bio-Techne, Minneapolis, MN), 1% sodium pyruvate (Gibco), 1% non-essential amino acids (Fisher Scientific, Waltham, MA), 1% sodium bicarbonate (Gibco), 1% penicillin streptomycin (Gibco), 1% L-glutamine (Gibco), 0.1% Amphotericin B, and 0.1% 2-mercaptoethanol (Gibco).

In Vitro Exposure to GWI Plasma

To evaluate the effect of chronic and acute GWI plasma on microglia, we used a modified protocol that was used in the Bose *et al.*, study (Bose *et al.*, 2020). Briefly, N9 microglia cells were seeded at 0.5×10^6 in a volume of 250 µL per well in a 48-well cell culture safe plate and allowed to rest for 24 hours. After 24 hours, media was replaced with a low-serum

media containing 1% FBS to simulate stress and to eliminate trophic factors. After an additional 24 hours (+48h), media was replaced with serum-free media and 2% of either GWI or control plasma was supplemented. Thirty minutes after this LPS (1 ng/mL) was added to select wells. The supernatant was collected 24 hours later and frozen at -20 °C for future analysis.

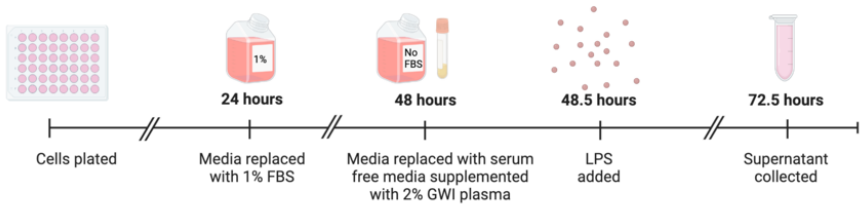


Figure 3.1. *In vitro* GWI Plasma experimental design.

GWI Plasma

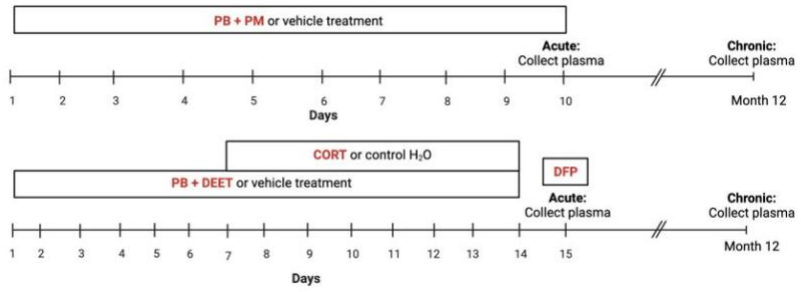


Figure 3.2. Acute and Chronic GWI Studies from which Plasma was Sourced

Acute plasma for this study was plasma from PB + PM or PB, DEET, CORT + DFP or vehicle-treated mice from the Carpenter *et al.* 2020 study. These groups will be referred to as PB + PM plasma and DFP plasma respectively. Briefly, the PB + PM plasma was from mice treated with PB (0.7 mg/kg IP) and PM (200 mg/kg IP) or DMSO vehicle for 10 days. DFP plasma was from mice treated with PB (2 mg/kg SQ), DEET (30 mg/kg SQ), or saline vehicle, followed by

CORT (200 mg/L in 1.5% EtOH water) or vehicle administered on days 7-14. Finally, a single injection of DFP (3.75 mg/kg) was given on day 15. Six hours after the last treatment on both models, intracardiac blood was taken before the mice were euthanized. Whole blood was spun down, and plasma was collected and frozen at -80°C for future use.

Chronic plasma was from an unpublished study that utilized the same exposure dosages and times as the acute study; however, the mice were allowed to age for 12 months after the GWC dosing. Plasma was collected in the same way as in the acute study.

Plasma from both acute and chronic models was screened for acute phase proteins, C-reactive protein (CRP) and serum amyloid A (SAA). To accomplish this, plasma was diluted to 1:10,000 in reagent diluent (1% BSA) for CRP, and 1:200 for SAA. Following this, ELISAs were performed using Duo-Set ELISA CRP and SAA kits (Bio-Techne, Minneapolis, MN). Samples were run in duplicate following the manufacturer's protocol.

Cytokine analysis

To evaluate the levels of cytokines in the media, ELISAs were performed using Duo-Set ELISA kits (Bio-techne, Minneapolis, MN). At the end of the exposure time, Hank's Buffer medium was collected and frozen for ELISA. Samples were run in duplicate following manufacturer's protocol. TNF-α was the inflammatory cytokine evaluated in this study.

Results

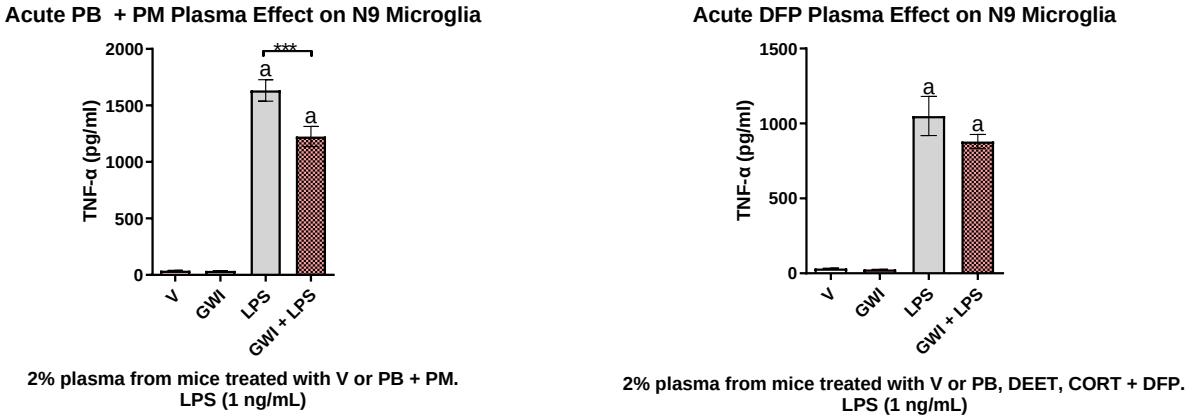
3.1.1. Acute phase protein analysis of chronic GWI plasma

CRP and SAA content in the chronic plasma was analyzed via ELISA and no significant differences between vehicle treated mice plasma and chronic GWI plasma for both models was

noted. There was a slight numerical increase in CRP in the DFP model plasma over the vehicle treated plasma (two tailed p value = 0.227, Figure 3.5). Since CRP and SAA were not elevated at the chronic timepoint, we elected not to evaluate the acute plasma as if these APPs changed acutely, these data demonstrated that this effect does not persist in mice.

3.1.2. Cytokine analysis on the effect of acute and chronic GWI plasma on N9 microglia

On its own, acute PB + PM plasma and acute DFP plasma did not cause a significant change in TNF-α production (Figure 3.4). Pretreatment with acute PB + PM model plasma resulted in a decrease in LPS induced TNF-α secretion ($p \leq 0.001$, Figure 3.4). Chronic PB + PM and DFP plasma did not cause a significant effect in TNF-α or IL-6 secretion in the absence of LPS (Figure 3.6 and 3.7). Chronic PB + PM plasma followed by an LPS challenge resulted in a significant increase in LPS induced TNF-α secretion ($p \leq 0.0$, Figure 3.6). The chronic DFP plasma had the opposite effect when followed by LPS with significant decreases in LPS induced TNF-α and IL-6 secretion occurring in N9 microglia that received chronic DFP plasma prior to LPS ($p \leq 0.001$, $p \leq 0.05$ respectively. Figure 3.7).



a. Acute PB + PM Plasma **b. Acute DFP Plasma**

Figure 3.3. Acute PB + PM and DFP models plasma effects on N9 microglia TNF- α production.

N9 cells were plated and allowed to settle in complete media for 24 hours. After 24 hours, media was replaced with a low-serum media containing 1% FBS to simulate stress and to eliminate trophic support. After an additional 24 hours (+48h), the media was replaced with serum-free media and 2% of either GWI or V plasma was supplemented. Thirty minutes later, LPS was added to select wells. Supernatant was collected after 24 hours this and frozen at -20 °C until cytokine analysis. Across both the DFP and PB + PM models, there was no significant difference noted between V and GWI plasma in the absence of LPS. However, with LPS, GWI plasma from both DFP and PB + PM mice caused N9 microglia to produce significantly less TNF-α when compared to vehicle-treated plasma + LPS. Data is presented as mean ± SEM. GWC effects are indicated by *** = $p \leq 0.00$. 'a' indicates a significant LPS effect at $p \leq 0.05$.

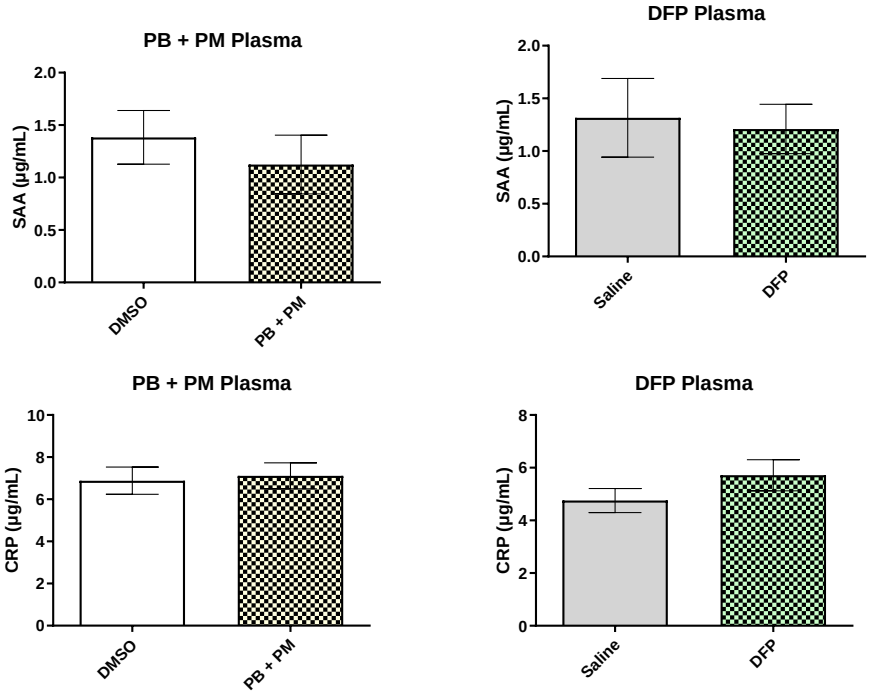
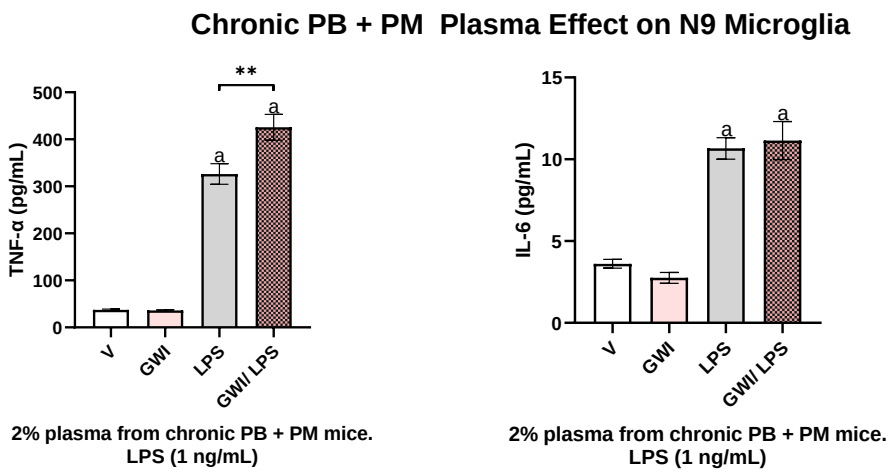


Figure 3.4. CRP and SAA levels in chronic PB + PM and DFP model plasma.

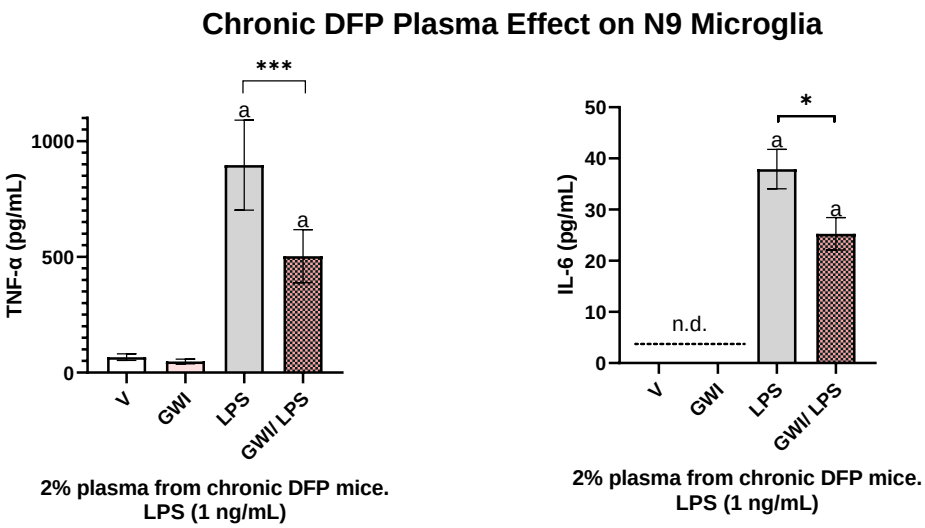
Chronic plasma from the unpublished Carpenter *et al.*, study was analyzed via ELISA for CRP and SAA. Plasma was either from a vehicle (saline or DMSO)-treated mouse or from a GWI-treated mouse (PB + PM/DFP models). Data shows no significant differences between vehicle or GWI treated mouse plasma levels of CRP and SAA. However, a slight but non-significant increase in CRP levels is present in plasma of mice treated with the DFP model. Data is presented as mean ± SEM.



a. Chronic PB + PM Plasma TNF- α **b. Chronic PB + PM Plasma IL-6**

3.5. Chronic PB + PM model plasma effect on N9 microglia TNF-α and IL-6 production.

N9 cells were plated and allowed to settle in complete media for 24 hours. After 24 hours, media was replaced with a low-serum media containing 1% FBS to simulate stress and to eliminate trophic support. After an additional 24 hours (+48h), the media was replaced with serum-free media, and 2% of either GWI or V plasma was supplemented. Thirty minutes later, LPS was added to select wells. The supernatant was collected after 24 hours and frozen at -20 °C until cytokine analysis. **A)** TNF-α levels showed no significant differences between vehicle-treated mice and GWI-treated mice. When cells were pretreated with 2% GWI or vehicle plasma and then challenged with LPS, a significant difference between treatment groups emerged, with chronic PB + PM plasma causing a significant upregulation in TNF- α. **B)** IL-6 analysis revealed no significant effects, aside from the response to LPS treatment. Data is presented as mean ± SEM. GWC effects are indicated by ** = $p \leq 0.01$. 'a' indicates a significant LPS effect at $p \leq 0.05$.



a. Chronic DFP Plasma TNF- α **b. Chronic DFP Plasma IL-6**

3.6. Chronic DFP model plasma effect on N9 microglia TNF- α and IL-6 production.

N9 cells were plated and allowed to settle in complete media for 24 hours. After 24 hours, the media was replaced with a low-serum media containing 1% FBS to simulate stress and to eliminate trophic support. After an additional 24 hours (+48h), the media was replaced with serum-free media, and 2% of either GWI or V plasma was supplemented. Thirty minutes later, LPS was added to select wells. The supernatant was collected after 24 hours and frozen at -20 °C until cytokine analysis. **A)** TNF- α levels showed no significant differences between vehicle-treated and GWI-treated mice. When cells were pretreated with 2% GWI or vehicle plasma and then challenged with LPS, the chronic DFP plasma caused a significant reduction in the LPS response over the vehicle-treated mice. **B)** These same effects were noted in IL-6 production with chronic DFP plasma resulting in a decreased inflammatory response of N9 microglial cells to LPS. Data is presented as mean ± SEM. GWC effects are indicated by *** = $p \leq 0.001$, * = $p \leq 0.05$. 'a' indicates a significant LPS effect at $p \leq 0.05$.

Discussion

Multiple studies have linked the symptoms of GWI with persistent inflammation within the periphery as well as in the CNS (Alshelh *et al.*, 2020; Carpenter *et al.*, 2020; Johnson *et al.*, 2016; Parihar *et al.*, 2013; Parkitny *et al.*, 2015). However, the precise mechanisms that cause inflammation in GWI are unknown. The present study sought to evaluate the neuroinflammatory mechanisms of GWI by analyzing the indirect effects of plasma from GWC-treated animals on microglial activation. This study implemented the use of two widely established GWI models, the O'Callaghan *et al.*, 2015 'DFP model' and the Zakirova *et al.*, 2015 'PB + PM model'.

It has been shown that low-grade peripheral inflammation can influence neuroinflammation resulting in subsequent neurological dysfunction (Cunningham & Hennessy, 2015). Veterans with GWI have been noted to have higher circulating levels of inflammatory factors such as cytokines, acute phase proteins, and other inflammatory proteins (Garza-Lombó *et al.*, 2021; Hodgin *et al.*, 2022; Johnson *et al.*, 2016). The link between the peripherally initiated inflammation and the neuroinflammatory symptoms of GWI is understudied. The present study sought to determine if the neuroinflammatory effects reported in GWI could be induced through peripheral influence. It has been speculated that peripheral inflammation may serve to initiate or exacerbate neuroinflammation, and this study hypothesized that this may occur by inducing microglial hyperactivation.

A study by Hodgin *et al.*, noted elevated inflammatory proteins in the serum of GWI patients. In particular, the APPs, CRP and SAA, were noted to be increased with the severity of symptoms (Hodgin *et al.*, 2022). APPs are produced by the liver as the result of an innate immune activation and can be indicative of a widespread inflammatory response (Schrodl *et al.*, 2016). To date, there is no widely accepted biomarker that can be used to monitor GWI effects

on both the periphery and CNS. While CRP and SAA have been investigated as potential biomarkers for GWI severity, the specific effects of GWC exposure paradigms on circulating APP levels is under evaluated. Both CRP and SAA were investigated in plasma from mice treated with PB + PM or PB/DEET/CORT/DFP (DFP model). CRP and SAA levels were not significant differences between the plasma of mice treated with the PB + PM or DFP models compared to plasma from vehicle-treated mice. It is possible that the murine GWI models may not produce the same significant differences noted in human veterans with GWI. Indeed, differences in APP concentrations between the human and the mouse inflammatory response have been noted (Lu *et al.*, 2018). Nevertheless, we sought to explore the effect of peripheral influence on neuroinflammation to see if GWI resulted in a peripherally driven neuroimmune response. While CRP and SAA did not appear to be significantly elevated in these models, it is possible that there may be other circulating factors that could exacerbate the inflammatory response in the CNS and/or be indicative of CNS inflammation and associated damage and dysfunction.

To investigate if the periphery could influence neuroinflammation in GWI, plasma from both the PB + PM and DFP models was applied to N9 microglial cells. The acute PB + PM plasma and the acute DFP plasma did not result in a significant change in inflammatory cytokine secretion when given without an inflammagen. However, when given prior to stimulating with LPS, acute plasma from the PB + PM model resulted in a significant downregulation in LPS-elicited TNF-α release from microglia. Likewise, a decrease in TNF-α production occurred when the acute DFP plasma was administered before LPS. The chronic PB + PM plasma produced no significant change on its own, but, when followed by LPS exposure, a significant increase in TNF-α and a slight increase in IL-6 was noted. Like the chronic PB + PM plasma, the chronic

DFP plasma did not change microglial activation in the absence of LPS. When applied prior to stimulating with LPS, the chronic DFP plasma led to a significant decrease in the inflammatory response.

These data show that while chronic GWI plasma did not have any significant changes in APPs, chronic PB + PM plasma still caused a hyperactivated response in microglia. On the other hand, the chronic DFP plasma resulted in a significant decrease in the inflammatory response. Taken together, these data suggest a mechanistic difference in induction of neuroinflammation of both models. The DFP model plasma, at both chronic and acute timepoints, has no inflammatory effect on microglia, implying that the mechanism of this model is likely non-humoral. Conversely, the PB + PM plasma results in an increase in microglial activation, suggesting a humoral connection, but only at the chronic timepoint. Acutely, the PB + PM plasma did not cause an increase in inflammation. Our laboratory has previously found the DFP model to result in more severe and widespread acute neuroinflammation than the PB + PM model (Carpenter *et al.*, 2020). However, the DFP model and components of this model have been shown to result in a lack of microglial inflammation (Koo *et al.*, 2018; O'Callaghan *et al.*, 2015). In human veterans, variation in symptom type and severity have been linked to differences in reported exposure conditions (Kerr, 2015). Circulating levels of acute phase proteins, cytokines, and other inflammatory analytes can vary in GW veterans based on their symptoms (Hodgin *et al.*, 2022). Taken together, it may be possible that differing GWC exposure paradigms result in varying inflammatory mechanisms within the periphery. These data also illustrate that microglial inflammatory changes occur not only based on chemical exposure, but also due to age. Only the chronic PB + PM model caused microglia to increase their inflammatory response. This correlates data that confirms the worsening of symptoms in GWI veterans as they age (Li *et al.*,

2011; RACGWI, 2014). Overall, our data confirms that the ability for peripheral inflammation to influence neuroinflammatory responses varies based on exposure conditions. This was clearly demonstrated by the DFP model plasma resulting in no significant difference in microglial activation through the humoral pathway. In contrast, plasma from the PB + PM model did show a humoral mechanism by causing an increase in microglial activation, resulting in elevated pro-inflammatory cytokine production. These data may help to explain why GWI symptoms can vary from veteran to veteran.

3b. The effect of GWC on *ex vivo* brain tissue and the inflammatory response.

Introduction

Currently, it is unknown whether GWI originates within the periphery, the CNS, or both. Within the CNS, microglia have been implicated as a causative agent in initiating neuroinflammation in many neurodegenerative diseases (McGeer & McGeer, 2002; Streit *et al.*, 2004). However, neurons and astrocytes have also been shown to influence the microglial inflammatory response (Baxter *et al.*, 2021). Astrocytes communicate with both neurons and microglia to regulate many neuroimmune functions, including upkeep of the BBB integrity (Liu *et al.*, 2020). It has been shown that astrocytes and neurons can influence microglial gene expression, prompting an increase in inflammatory or anti-inflammatory genes (Baxter *et al.*, 2021). It is possible that microglia may not be directly impacted by GWC; rather, these chemicals may activate other neuroimmune cells that then trigger microglial activation. Studies have shown that GWI invokes a more robust increase in astrocyte activation when compared to microglia (Carpenter *et al.*, 2021). Astrocytes activated by GWC can become hypertrophic and cause a decrease in neurogenesis and increased neuronal loss (Abdullah *et al.*, 2011; Bryant *et al.*, 2021; Madhu *et al.*, 2021; Parihar *et al.*, 2013).

To further explore the neuroimmune response, this study investigated the effects of GWC on brain tissue and the influence of these chemicals on the inflammatory response within specific regions of the brain. By exposing brain slices to GWC, the impact of GWC on specific cell types

within regions of interest could be studied. To elucidate the effects, the impact of GWC on neuroinflammation were assessed regionally via cytokine analysis. This study analyzed the tissue response in the striatum as well as the hippocampus, two areas that have been shown to be heavily affected in GWI (Abdel-Rahman et al., 2004; Brown et al., 2021; Chao et al., 2017; Miller et al., 2018). The purpose of this chapter was to determine whether GWC indirectly increases inflammatory dysregulation through a tissue specific response. By using *ex vivo* slicing techniques, we can more accurately determine the specific regional effects of GWC.

Materials and Methods

Gulf War Chemicals

N-N, Diethyl-3-methylbenzamide (DEET, Millipore Sigma, St. Louis, MO), permethrin (PM; 28% cis/71% trans isomer; 99% purity; ChemServices Inc., West Chester, PA), pyridostigmine bromide (PB; ≥ 98% purity; Sigma Aldrich, St. Louis, MO), diisopropylfluorophosphate (DFP, Sigma Aldrich), and corticosterone (CORT; Steraloids, Newport, RI) were used. DFP served as a sarin surrogate, while CORT was utilized to simulate war-theater stress.

Animals

Male C57BL/6 mice (ranging from 18 to 26 months old, Jackson Laboratories, Bar Harbor, ME) were housed in an environmentally controlled room and maintained on a 12-h light/dark cycle with food and water available *ad libitum*. Procedures were approved in advance by the University of Georgia Institutional Animal Care and Use Committee (IACUC).

Ex vivo live slice experimentation

Following CO_2 euthanasia, brains were quickly removed, rinsed, and placed in ice-cold oxygenated Hank's-buffered solution (HBSS; pH 7.4). Brains were sliced into 350 µm thick sections using an oscillating tissue slicer (EMS-4000, Electron Microscopy Sciences, Hatfield, PA). Immediately following the sectioning, 2-mm diameter punches of striatal tissue were collected bilaterally with 8 striatal punches taken per animal. Hippocampal slices were bisected, and each half was collected with 6 slices total taken per animal. The tissue was then placed into 24-well plates containing 0.5 mL of Hank's Buffer supplemented with 1% horse serum (Figure 3.3). Plates were placed in a Dubnoff metabolic shaker bath (Precision Scientific Inc. Chicago, IL), shaken at 80 oscillations per min at 37°C temperature under an atmosphere of 95% O_2 and 5% CO_2, and allowed to rest for 30 minutes. After this, GWC treatment was added, and plates were incubated in the metabolic shaker bath for the duration of the exposure time. After exposure, both the tissue and supernatant were collected and frozen at -20 °C for further analysis.

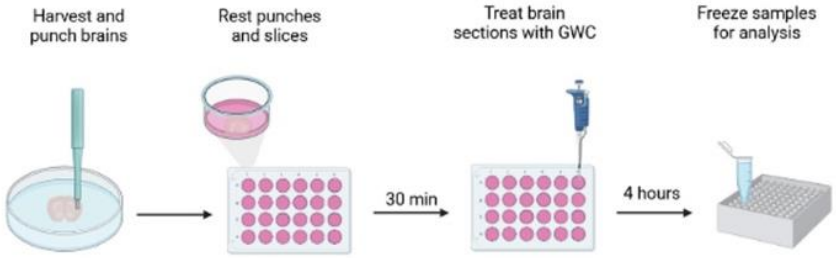


Figure 3.7. *Ex Vivo* Experimental Design

Cytokine analysis

To evaluate the levels of cytokines in the media, ELISA was performed using Duo-Set ELISA kits (Bio-techne, Minneapolis, MN). At the end of the exposure time, Hank's Buffer medium was collected and frozen at -20 °C for ELISA. Samples were run in duplicate following manufacturer's protocol. TNF-α was the inflammatory cytokine evaluated in this study.

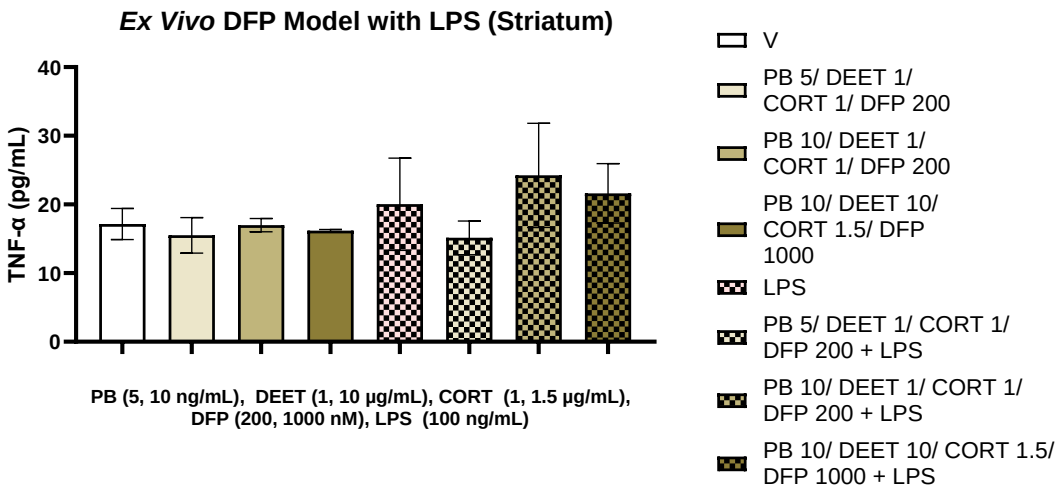
Results

3.2.1. Cytokine analysis for the DFP model

Two different experimental protocols were performed for the DFP model. In the first experimental protocol (refer to figure description for details), no significant changes in TNF-α secretion that could be attributed to GWC treatment were noted in the striatum and hippocampus (Figure 3.8 and 3.9). In the second experiment we increased the dose and duration of LPS exposure. In the striatum, no significant changes attributed to GWC treatment were noted (Figure 3.12). In the hippocampus, both doses of the DFP model caused a significant decrease in LPS induced TNF-α secretion ($p \leq 0.01$, $p \leq 0.05$, Figure 3.13) suggesting that acutely this model does not induce neuroinflammation within the tissue.

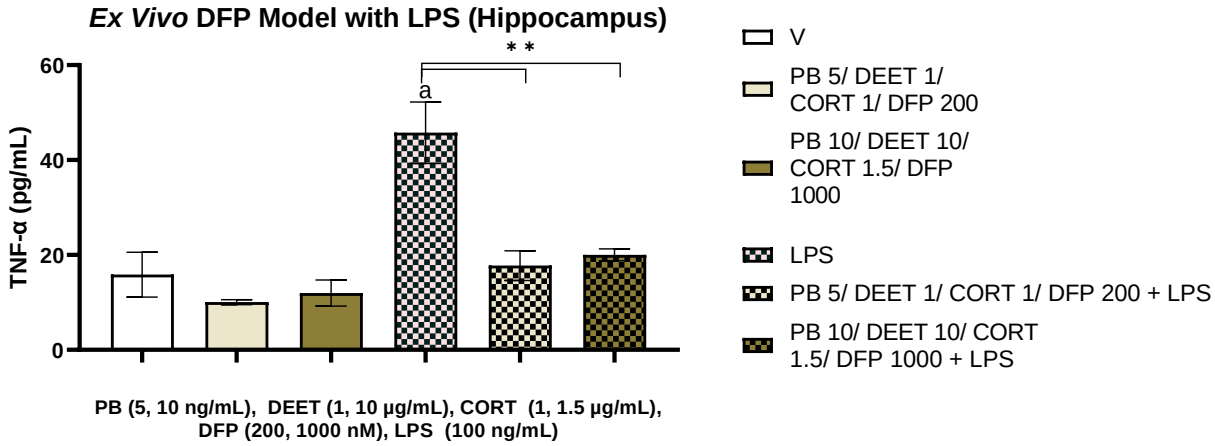
3.2.2. Cytokine analysis for the PB + PM model

In the striatum, the PB + PM model did not cause significant increases in TNF-α secretion in the presence of absence of LPS (Figure 3.10). In the hippocampus, the PM on its own caused a significant decrease in TNF-α secretion ($p \leq 0.05$, Figure 3.11). When the PB + PM model was administered prior to an LPS challenge, PM 5 and 25 μM combined with PB 5 and 25 ng/mL resulted in a significant decrease in TNF-α levels ($p \leq 0.05$, Figure 3.11).



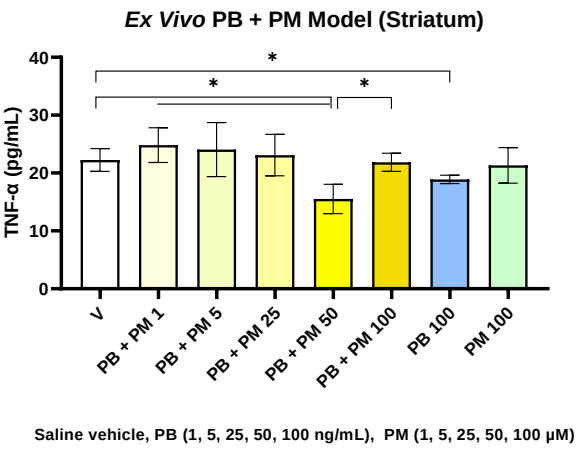
3.8: TNF-α Levels in *Ex Vivo* Striatal Punches Exposed to the DFP Model and 100 ng/mL of LPS.

Striatal punches were exposed to PB (5 or 10 ng/mL), DEET (1 or 10 µg/mL), CORT (1 or 1.5 µg/mL), and DFP (200 or 1000 nM) for 2 hours. After this pretreatment, LPS (100 ng/mL) was added to select samples, and all punches were incubated for an additional 2 hours. The supernatant was then collected and used for TNF-α analysis. No significant difference between treatments was noted. GWC did not result in a significant change in TNF-α in the striatum on its own or when LPS was administered. Data is presented as mean ± SEM.

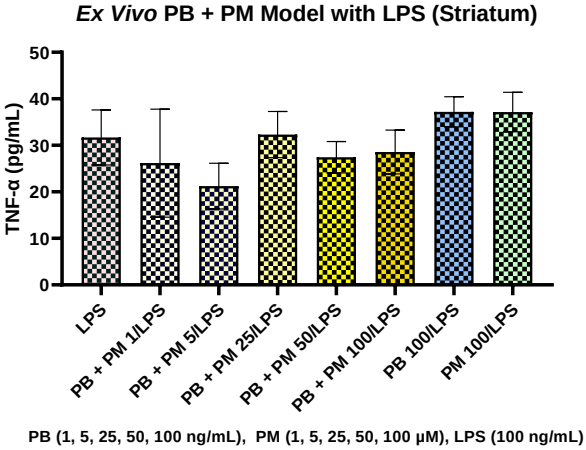


3.9. TNF-α Levels in *Ex Vivo* Hippocampal Slices Exposed to the DFP Model and 100 ng/mL of LPS.

Hippocampal slices were exposed to PB (5 or 10 ng/mL), DEET (1 or 10 µg/mL), CORT (1 or 1.5 µg/mL), and DFP (200 or 1000 nM) for 2 hours. After this pretreatment, LPS (100 ng/mL) was added to select samples, and all slices were incubated for an additional 2 hours. Supernatant was then collected and used for TNF-α analysis. GWC had no effect in TNF-α secretion in the hippocampus on its own or when LPS was administered. LPS caused a significant increase in TNF-α, but this effect was not exacerbated by GWC. Data is presented as mean ± SEM. GWC effects are indicated by ** = $p \leq 0.01$. 'a' indicates a significant LPS effect at $p \leq 0.05$.



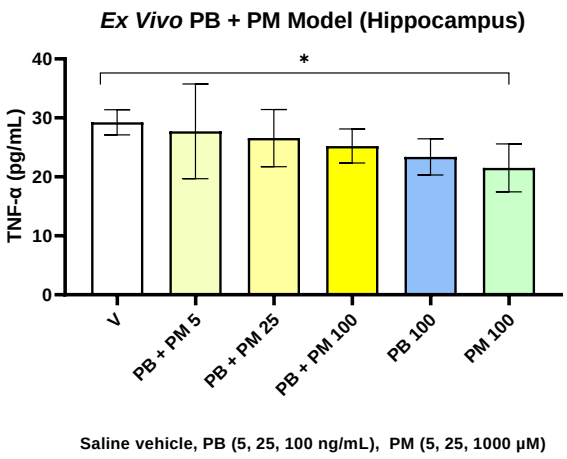
a. PB + PM treatment



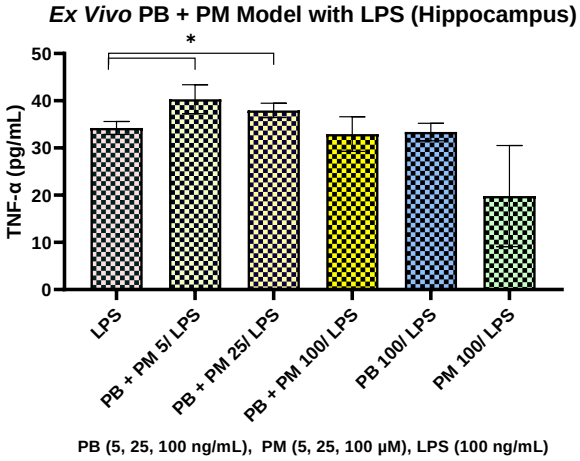
b. PB + PM treatment with LPS

3.10. TNF-α Levels in *Ex Vivo* Striatal Punches Exposed to the PB + PM Model.

Striatal punches were exposed to PB (1, 5, 25, 50 or 100 ng/mL) and with PM (1, 5, 25, 50 or 100 µM) for 2 hours. After this pretreatment, LPS (100 ng/mL) was added to select samples, and all punches were incubated for an additional 2 hours. Supernatant was then collected and used for TNF-α analysis. a) PB and PM given together or separately do not cause a significant change in inflammation in the striatum. b) PB and PM given together or separately followed by an inflammatory challenge do not cause a significant effect in inflammation in the striatum. Data is presented as mean ± SEM. GWC effects are indicated by * = $p \leq 0.05$.



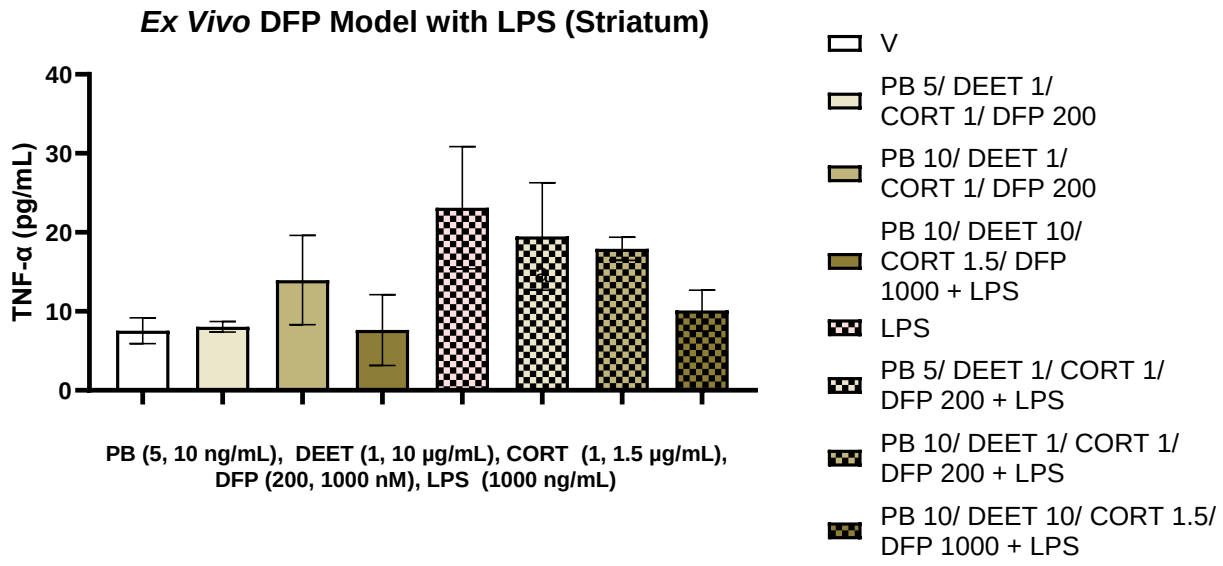
a. PB + PM treatment



b. PB + PM treatment with LPS

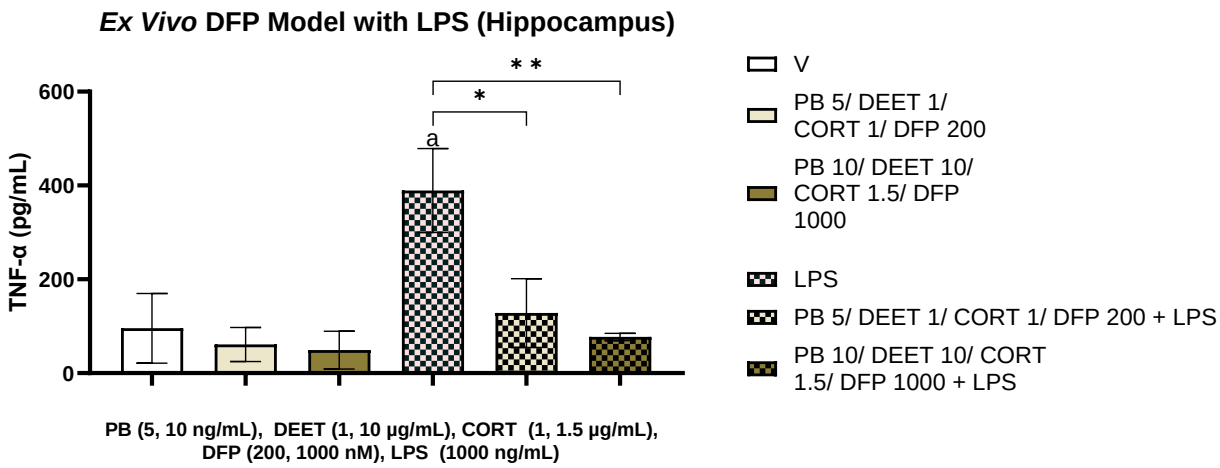
3.11. TNF-α Levels in *Ex Vivo* Hippocampal Slices Exposed to the PB + PM Model.

Hippocampal slices were exposed to PB (1, 5, 25, 50 or 100 ng/mL) and with PM (1, 5, 25, 50 or 100 µM) for 2 hours. After this pretreatment, LPS (100 ng/mL) was added to select samples, and all punches were incubated for an additional 2 hours. The supernatant was then collected and used for TNF-α analysis. **a)** PB and PM given together or separately do not cause a significant effect in inflammation in the hippocampus. **b)** PB + PM 5 and PB + PM 25 given before LPS cause a significant increase in TNF-α produced within the hippocampus. Data is presented as mean ± SEM. GWC effects are indicated by * = $p \leq 0.05$.



3.12: TNF-α Levels in *Ex Vivo* Striatal Punches Exposed to the DFP Model and 1000 ng/mL of LPS.

Striatal punches were exposed to PB (5 or 10 ng/mL), DEET (1 or 10 µg/mL), CORT (1 or 1.5 µg/mL), and DFP (200 or 1000 nM) for 1 hour. After this pretreatment, LPS (1000 ng/mL) was added to select samples, and all punches were incubated for an additional 3 hours. Supernatant was then collected and used for TNF-α analysis. There was no significant effect of GWC pretreatment in the striatum. A non-significant decrease in TNF-α could be noted in striatal punches treated with the DFP model followed by LPS administration. Data is presented as mean ± SEM.



3.13. TNF-α Levels in *Ex Vivo* Hippocampal Slices Exposed to the DFP Model and 1000 ng/mL of LPS.

Hippocampal slices were exposed to PB (5 or 10 ng/mL), DEET (1 or 10 µg/mL), CORT (1 or 1.5 µg/mL), and DFP (200 or 1000 nM) for 1 hour. After this pretreatment, LPS (1000 ng/mL) was added to select samples, and all slices were incubated for an additional 3 hours. Supernatant was then collected and used for TNF-α analysis. GWC did not result in a significant change in TNF-α in the hippocampus on its own or when LPS was administered. LPS caused a significant increase in TNF-α, but this effect was influenced significantly by GWC. Both doses of the DFP model caused a significant decrease in the inflammatory effect of LPS. Data is presented as mean ± SEM. GWC effects are indicated by ** = $p \leq 0.01$, * = $p \leq 0.05$. 'a' indicates a significant LPS effect at $p \leq 0.05$.

Discussion

To further understand the effect of GWC on neuroinflammation, *ex vivo* slice exposure paradigm was used. In the literature, both GWI models have been shown to have hippocampal and striatal aberrations, including structural, neurochemical, and neuroinflammatory repercussions, as a result of GWC exposure (Abdel-Rahman *et al.*, 2004; Brown *et al.*, 2021; Carpenter *et al.*, 2020; Chao *et al.*, 2017; Miller *et al.*, 2018). By utilizing an *ex vivo* model, the effect of each exposure paradigm could be investigated in a dose-dependent manner. The effects of the PB + PM and the DFP model were investigated on striatal and hippocampal slices. *Ex vivo* brain tissue to study the effects of GWI is not something that has been widely documented in the available literature. Part of this aim sought to develop an appropriate protocol for GWC exposure in *ex vivo* slices. With our first protocol, neither the PB + PM nor the DFP model in the absence of LPS caused any changes in the secretion of the cytokines that were measured. When the PB + PM model was given prior to LPS, the two lower concentrations did result in an upregulation in TNF-α within the hippocampus. Following the first set of *ex vivo* slice experiments, we modified our protocol to extend GWC exposure duration and increase the LPS concentration. In the striatum, no significant effect of GWC occurred. However, both doses of the DFP model caused a dose dependent-decrease in LPS-induced inflammation. Thus, in the hippocampus only, components of DFP model, when applied acutely, had an anti-inflammatory effect on TNF-α production.

Several studies have highlighted hippocampal inflammation and dysfunction in veterans suffering from GWI (Apfel *et al.*, 2011; Chao *et al.*, 2017; Menon *et al.*, 2004). This study sought to investigate the inflammatory response to varying GWC models and doses to determine the extent of the inflammatory effect. An increase in inflammation was only seen when

hippocampal slices received PB + PM pretreatment before LPS challenge. This effect was dose-dependent, and only occurred at the two lower doses of the PB + PM model used. This suggests that, on its own, short-term exposure to the PB + PM model without any additional stimulation does not cause inflammation in the hippocampus. However, if PB + PM exposure occurs before an inflammatory stimulus, a priming effect occurs, which causes an exaggerated inflammatory response. The priming effect is dose dependent and did not occur at the highest dose of PB + PM used, or when PB and PM were used separately. The hippocampus has been shown to be densely populated with microglia (Ochocka & Kaminska, 2021), a cell type that we have shown responds to the PB + PM model with an increase in LPS-stimulated inflammatory cytokine secretion. We have shown in the previous chapters that microglia can be primed for an increased inflammatory response with the PB + PM model. The prevalence of microglia in the hippocampus may account for the increase in GWC-driven neuroinflammation seen in the PB + PM model.

Interestingly, the DFP model has been previously noted to produce significant inflammatory changes in the hippocampus in *in vivo* studies (Carpenter *et al.*, 2020). This varied from our *ex vivo* findings, which showed that when combined with a high dose of LPS (1000 ng/mL), the DFP model caused a significant decrease in inflammatory cytokine production in the hippocampus in an *ex vivo* setting. This may be due to the direct application of the model to the brain tissue and the acute nature of this study. Corticosterone, one of the components of the DFP model, has been shown to have anti-inflammatory effects *in vitro* during short term exposure (Liu *et al.*, 2018); the reduction of LPS-induced inflammation in the hippocampal slices from the DFP model observed in our study might have been the result of the acute anti-inflammatory effects of corticosterone, increased acetylcholine (due to DFP), or both.

In terms of the striatal response, the DFP model has been noted to result in striatal increases in TNF-α *in vivo* (Miller *et al.*, 2018). In our study, a striatal DFP effect was also not noted in either DFP model from this study. However, a slight decrease in TNF-α occurred in the striatal slices that received DFP model pre-treatment for 1 hour followed by a challenge with 1000 ng/mL of LPS. This data mirrors the DFP hippocampal data that showed a similar, but significant, decrease in LPS-induced inflammation in slices that received DFP model pre-treatment. For the PB + PM model, no significant increases in inflammation occurred in the striatal punches in the presence of PB + PM alone or when it was combined with LPS.

Collectively, the data from this subchapter underscores the complicated nature of these models. When applied directly to brain tissue, only the hippocampal tissue responded to GWC and only to GWC that were applied prior to LPS. The PB + PM model yielded an increase in inflammatory cytokine secretion, while the DFP model resulted in a decrease in TNF-α and only after an increased dose of LPS. Together, these data help to illustrate the regional tissue response differences in GWI and suggest that PB + PM exposure may result in hippocampal initiation of neuroinflammation. On the other hand, the DFP model of neuroinflammation does not cause neuroinflammation through direct tissue damage in the hippocampus or striatum. These data comply with what has been noted in the literature, wherein the DFP model resulted in global neuroinflammation in the absence of tissue damage (O'Callaghan *et al.*, 2015). It is possible that, acutely, the DFP model results in an anti-inflammatory effect, but chronically, or in combination with aging, the effect becomes pro-inflammatory. Nevertheless, we showed that acute regional exposure of the DFP model does not result in increased tissue inflammatory response in both the striatum and hippocampus.

Moving forward, *ex vivo* slicing could further be used to evaluate the *in vivo* repercussions of GWI on specific cell types as well as the tissue as a whole. GWI has been linked to decreased neurogenesis, neuronal decrease, astrogliosis, and decreases in oligodendrocyte proliferation (Abdullah *et al.*, 2011; Belgrad *et al.*, 2019; Bryant *et al.*, 2021; Madhu *et al.*, 2021; Parihar *et al.*, 2013). This study did not conduct staining to determine the effects of GWC on cell viability, proliferation, and distribution. In addition, qPCR may be more useful to determine cytokine mRNA trends, as it has been shown that mRNA production has been found to peak between 4 and 8 hours post stimulus whereas cytokine secretion may not peak until 8 to 24 hours after stimulus (Esnault *et al.*, 1996; O'Mahony *et al.*, 1998; Than *et al.*, 1997). The methodology developed in this study could be used to further evaluate the effects of GWC exposure regionally to further understand the pathogenesis of GWI.

Thesis Conclusions

Gulf War Illness (GWI) continues to negatively affect the lives of the veterans who were deployed during the Persian Gulf War over 30 years ago. Veterans with GWI suffer from multi-symptomatic issues resulting from immune system dysfunction, systematic inflammation and neuroimmune aberrations. Though decades have passed since the Gulf War, no widely accepted curative measures have been elucidated. A lack of viable therapeutic options is due, in part, to the complicated pathogenesis of the disease. GWI is thought to be a result of exposure to a combination of substances during the war. Pesticides, insect repellents, war theater stress, prophylactic medications, and, in a subset of troops, nerve gas agent exposure may culminate to produce the effects of GWI. Collectively, these agents are known as the Gulf War Chemicals (GWC), and the effect of exposure to these chemicals is widely studied as a causative factor behind GWI.

The goal of this study was to broaden the understanding of how GWI impacts neuroimmune functionality and neuroinflammation. To do this, we analyzed the effect of GWC on microglial cells. Microglia are the resident innate immune cell of the CNS, and they are associated with regulating inflammatory and homeostatic functions within the brain. Microglial dysfunction has been cited as a key factor in the pathology of many neurodegenerative diseases by causing unregulated neuroinflammation. Few studies exist that specifically explore the role of microglial dysfunction in GWI. This study aimed to explore the effects of two commonly used GWI models on microglial inflammation. The effects of the PB + PM model and the PB/DEET/CORT/DFP model (DFP model) on microglial activation were further characterized in this study.

In the second chapter, we analyzed the effect of direct application of GWC on microglial inflammatory cytokine production. We looked at the effects of the PB + PM and DFP models, as well as the individual chemicals from each model. We demonstrated that the PB + PM model and the DFP model have differing effects on microglial activation. In the absence of an additional inflammatory stimulus, neither model resulted in an inflammatory effect. When microglia were pretreated with the PB + PM model, and exposure ended prior to inflammatory stimulus with LPS, this model resulted in a potentiating effect with TNF- α. The same did not apply if the PB + PM treatment was not removed prior to LPS administration. With Positron Emission Tomography and MRI imaging, increased microglial activation and neuroinflammation have been noted in some veterans with GWI (Alshelh *et al.*, 2020). The PB + PM model has previously been associated with microglial activation and inflammation in rodent models (Zakirova *et al.*, 2016; Zakirova *et al.*, 2015). This study demonstrated that exposure to the PB + PM model leads to microglial priming and subsequent neuroinflammation.

Conversely, the DFP model had an anti-inflammatory effect, which reduced LPS-induced inflammation. This was shown to likely be a result of CORT, which we demonstrated to result in a dose-dependent decrease of inflammatory cytokine production. These data agree with the literature that has linked the DFP model to a lack of inflammatory microglial activation and an increase in M2-like markers (Carpenter *et al.*, 2020; O'Callaghan *et al.*, 2015) This implies that the DFP model does not initiate neuroinflammation through microglial hyperactivation. Together, these data proved that, when applied directly, the PB + PM model led to a microglial priming effect, while the DFP model resulted in an immunosuppressive effect in these cells. This illustrated that the differences in the type of GWC exposure experienced by the troops during the Gulf War may contribute to vastly different microglial effects. The differences in GWC exposure

may be responsible for the varying symptom severity noted in GWI, and thus, different therapeutic targets may be advantageous depending on the exposure risks of the GW veterans.

In the third chapter of this study, we analyzed the effects of peripheral influence on microglial activation. This indirect connection between the periphery and neuroinflammation in GWI has not been widely reported in literature. This study utilized both acute and chronic plasma from mice treated with the PB + PM model and the DFP model. Upon analyzing the chronic plasma for acute phase protein levels, it was found that neither C-reactive protein (CRP) nor serum amyloid A (SAA) were significantly affected by GWC treatment. However, a slight but non-significant increase in circulating CRP was noted in the chronic DFP model.

The effect of the peripheral influence of GWI on neuroinflammation was then tested by applying mouse model plasma to microglia followed by an inflammatory challenge. It was found that acutely, neither model resulted in a significant increase in microglial activation in the presence or absence of inflammatory stimulation. However, chronic PB + PM plasma yielded a significant potentiation effect to the inflammatory response of microglia. These data illustrate a mechanistic difference between the influence of peripheral inflammation on neuroinflammatory effects in GWI. PB + PM demonstrated a humoral effect, while the DFP model did involve a humoral mechanical. Collectively, this study confirmed the ability for peripheral inflammation to influence microglial inflammatory response in the PB + PM model of GWI. Studies have determined that circulating acute phase proteins and other inflammatory factors can vary in GW veterans and are linked the type and severity of self-reported symptoms (Hodgin *et al.*, 2022). The results of this study indicate that the type of GWC exposure that veterans may have experienced can result in differences in peripheral inflammation, which, in the PB + PM model, can contribute to neuroinflammation via humoral effects.

In the second part of the third chapter, we investigated the regional neuroinflammatory response to GWC treatment. To do this, an *ex vivo* slicing technique was used to assess the effect of GWC on striatal and hippocampal tissue. Both the DFP and PB + PM models have been linked to striatal and hippocampal aberrations (Abdel-Rahman *et al.*, 2004; Brown *et al.*, 2021; Carpenter *et al.*, 2020; Miller *et al.*, 2018). An increase in inflammatory cytokine secretion was seen in hippocampal, but not the striatal, slices that were exposed to PB + PM prior to an LPS challenge. These data suggest that acute exposure to PB + PM is enough to induce hippocampal imitation of neuroinflammatory changes.

The DFP model caused a significant decrease in the inflammatory response in the hippocampus but not the striatum when followed by a physiologically high dose of LPS. Studies have linked the DFP model to significant inflammatory changes in the rodent hippocampus and striatum (Carpenter *et al.*, 2020; Miller *et al.*, 2018). Despite significant increases in global neuroinflammation, the DFP model has also been associated with a lack of gliosis and neural death. It is possible that the DFP model results in an anti-inflammatory effect acutely but can become inflammatory with chronic exposure or in combination with the aging process. As a whole, these data further highlight the mechanistic differences in the neuroinflammatory effects of both GWC models.

Collectively, this thesis provided further context for the effect of the role of microglial activation in GWI. The PB + PM model has direct and indirect microglial priming effects, while the DFP model does not have humoral contributions or direct inflammatory effects on microglia. The exposure conditions that the veterans experienced during the war may play a significant impact on their disease development. The data from this study demonstrate the role of microglia in GWI neuroinflammation. By understanding the cellular contributions that microglia make to

the etiology of GWI, we have identified potential new avenues for therapeutic targets that could help to improve the quality of life for Gulf War veterans.

Supplementary Tables:

Results summary:

Chapter 2:

Direct effect of GWC *in vitro* on N9 microglia cytokine secretion

Treatment		PB (5 ng/mL) + PM (5 µM/mL)	PB (10 ng/mL) + PM (10 µM/mL)	PB (5 ng/mL) + PM (5 µM/mL) (IVIVE)	PB (10 ng/mL) + PM (10 µM/mL) (IVIVE)	PB (20 ng/mL) + PM (20 µM/mL) (IVIVE)
TNF-α	V	-	-	-	-	-
	LPS	↓*	↓*	-	-	↑***
IL-6	V	-	-	-	-	-
	LPS	-	-	-	-	-

S 2.1: PB + PM model *in vitro* summary

Treatment		CORT (0.5, 1, 1.5 µg/mL)	DEET (0.1, 1, 10 µg/mL)	DFP (20, 200, 1000 nM) (IVIVE)	CORT (1 µg/mL), DFP (200, 1000 nM) (IVIVE)	PB (5, 10 ng/mL), DEET (1, 10 µg/mL), CORT (1, 1.5 µg/mL) DFP (200, 1000nM) (IVIVE)
TNF-α	V	-	↓**,↓**,↓**	-	-	↓** (PB 10, DEET 1, CORT 1, DFP 200), ↓** PB 10, DEET 10, CORT 1.5, DFP 1000),
	LPS	↓***,↓***,↓***	↓*** (10)	↓* (200)	↓*** (CORT 1), ↓*** (CORT 1, DFP 200), ↓*** (CORT 1, DFP 1000)	↓***,↓***,↓***
IL-6	V	-	-	-	-	-
	LPS	↓***,↓***,↓***	↓*,↓*,↓*	-	-	-

S 2.2: DFP model *in vitro* summary

Chapter 3a:

Treatment		Acute PB + PM Plasma	Acute DFP Plasma	Chronic PB + PM Plasma	Chronic DFP Plasma
TNF-α	V	-	-	-	-
	LPS	↓***	-	↑**	↓***
IL-6	V	-	-	-	-
	LPS	-	-	-	↓*

S 3.1: Chronic and acute PB + PM and DFP model plasma effect on N9 microglia cytokine secretion

Treatment	Acute PB + PM Plasma	Acute DFP Plasma
CRP	-	-
SAA	-	-

S 3.2: Chronic PB + PM and DFP model plasma CRP and SAA levels

Chapter 3b:

The effect of GWC on *ex vivo* slices.

Treatment		**PB** (1, 5, 25, 50, 100 ng/mL), **PM** (1, 5, 25, 50, 100 µM) **Striatum**	**PB** (5, 25, 100 ng/mL), **PM** (5, 25, 100 µM) **Hippocampus**	**PB** (5, 10 ng/mL), **DEET** (1, 10 µg/mL), **CORT** (1, 1.5 µg/mL) **DFP** (200, 1000nM) **Striatum**	**PB** (5, 10 ng/mL), **DEET** (1, 10 µg/mL), **CORT** (1, 1.5 µg/mL) **DFP** (200, 1000nM) **Hippocampus**
TNF-α	V	↓* (PB 100) ↓* (PB + PM 50)	↓* (PM 100)	-	-
	LPS	-	↑*(PB+ PM 5), ↑* (PB+ PM 25)	-	-

S 3.3: PB + PM model and DFP model experiment 1

Treatment		**PB** (5, 10 ng/mL), **DEET** (1, 10 µg/mL), **CORT** (1, 1.5 µg/mL) **DFP** (200, 1000nM) **Striatum**	**PB** (5, 10 ng/mL), **DEET** (1, 10 µg/mL), **CORT** (1, 1.5 µg/mL) **DFP** (200, 1000nM) **Hippocampus**
TNF-α	V	-	-
	LPS	-	↓* (PB 5, DEET 1, CORT 1, DFP 200), ↓** (PB 10, DEET 10, CORT 1.5, DFP 1000)

S 3.4: DFP model experiment 2

'*' notes a significant GWC effect.

↓ = Decrease

↑ = Increase

- = No significant effect or cytokine not analyzed

*** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$

Figure acknowledgement

All non-data figures were generated with BioRender.com.

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