

Human Leukocyte Antigen (HLA) Associations and Gulf War Illness (GWI)

A Thesis
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Jasmine E. Joseph

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Adviser: Apostolos P. Georgopoulos MD, PhD
Co-adviser: Cavan S. Reilly PhD

January 2016

Acknowledgements

I would like to thank many people who have helped me along the way.

First, I would like to thank my advisor, Apostolos P. Georgopoulos, MD, for guiding, supporting and funding me over the years. Thank you, I have learned so much from you!

Second I would like to thank my committee members, Lisa M. James, PhD, and Cavan S. Reilly, PhD for their continuous support and encouragement in this project.

Finally, I would like to thank my family. My parents and siblings with their unconditional support helped me over come many obstacles, and made it easier to get back up when I fall. My husband Chandra moved to Minneapolis to be with me. He celebrated all my successes, encouraged me when I felt down, and made sure I never felt alone. I could not have done this without him. I have my family to thank for everything I am and what I achieved.

Dedication

To my husband, Nalinichandra Penke.

Abstract

Gulf War Illness (GWI) is a multi-symptom disease of unknown etiology that was observed in veterans after the first Gulf War (1990-1991). GWI involves multiple organ systems and has symptoms suggesting the involvement of the immune system. Many studies show Human Leukocyte Antigen (HLA) associations with autoimmune, infectious, and inflammatory diseases. Researchers have hypothesized that the multi-symptom nature of GWI may share an autoimmune component. In order to test for a potential immunological component in GWI, we test the hypothesis that HLA might differ between veterans with GWI compared to Gulf War era veterans without GWI.

Table of Contents

Contents

Acknowledgements.....	i
Dedication.....	ii
Abstract.....	iii
Table of Contents.....	iv
List of Tables.....	vi
List of Figures.....	vii
Gulf War Illness Overview.....	1
Case Definition.....	1
What Caused Gulf War Syndrome?.....	3
HLA Overview.....	5
HLA nomenclature.....	6
HLA and Other Diseases.....	7
HLA Class I Disease Mechanisms.....	8
HLA Class II Disease Mechanisms.....	9
Research Goal.....	10
Study Design.....	10
Materials and Methods.....	11
Gulf War Veteran Population [Disease and Control Population].....	11
HLA Genotyping.....	11
Data Analysis.....	13
Classification.....	13
Allele Effect: Protective or Predisposing.....	13
Symptom Domains affected by HLA.....	14
Comparing allele frequencies to other databases.....	14
Results.....	15
Classification.....	15
Alleles protective or predisposing.....	17
Symptom Domains affected by HLA.....	18

Comparing allele frequencies to other databases	19
Discussion	22
Bibliography	25

List of Tables

Table 1 Demographic information 10

Table 2 Results Stepwise Linear Discriminant Analysis (LDA)..... 15

Table 3 Classification Results 16

Table 4 Frequency and Odds Ratio 17

List of Figures

Figure 1	7
Figure 2	18
Figure 3	20
Figure 4	20
Figure 5	21

Gulf War Illness Overview

The Gulf War (1990–1991) was an international conflict triggered by Iraq’s invasion of Kuwait on August 2, 1990. About 700,000 military personnel participated in the Gulf War to liberate Kuwait from Iraqi troops (VA, 2012a). After the war, several thousand veterans complained of various unexplained symptoms such as chronic fatigue, rash, headache, arthralgia/myalgia, difficulty concentrating, forgetfulness, and irritability. The term Gulf War Illness (GWI) was first used to describe these chronic unexplained symptoms (Fukuda et al., 1998). “These symptoms have not been localized to any one organ system, and there has been no consistent physical sign or laboratory abnormality that indicates a single specific disease” (Blanck, 1995). The estimate of the Gulf War (GW) veterans who have GWI range from 175,000 to 250,000 (about 25–35% of the GW veteran population)(Institute of Medicine National Research Council, 2010). Studies have shown that GW veterans report these numerous chronic nonspecific symptoms significant more often than their non-deployed peers (Fukuda et al., 1998).

Case Definition

There are two case definitions for GWI: the Fukuda/CDC case definition and the Steele/Kanas case definition. The Fukuda/CDC case definition of GWI is when a veteran reports one or more symptoms present for 6 months or longer from at least two of the following three categories (Fukuda et al., 1998):

- Fatigue
- Mood and cognition (symptoms of feeling depressed, difficulty in remembering or concentrating, feeling moody, feeling anxious, trouble finding words, or difficulty sleeping)
- Musculoskeletal (symptoms of joint pain, joint stiffness, or muscle pain)

The Steele/Kanas Gulf War Illness (GWI) case definition was developed from a population-based questionnaire based on health and exposure questions of 2,000 Kanas Veterans who served during the 1990-1991 Gulf War (Steele, 2000). The Fukuda/CDC criteria is less restrictive (broader) and served as the initial base for screening veterans for the Steele/Kanas GWI definition. The Steele/Kanas GWI definition has exclusionary and inclusionary components (Steele, 2000). Veterans are excluded from consideration if they have been diagnosed with a chronic medical condition or a psychiatric condition that interferes with the veteran's ability to report symptoms. Veterans are included for consideration of GWI if they have multiple and/or moderate to severe chronic symptoms in at least three of six defined symptom domains (described below). Symptoms must have first been a problem during or after the Gulf War and persisted over the 6-month period.

The six symptom domains include (Steele, 2000):

- Fatigue/sleep problems
- Somatic pain
- Neurologic/cognitive/mood symptoms
- Gastrointestinal symptoms
- Respiratory symptoms

- Skin abnormalities

What Caused Gulf War Syndrome?

There were many factors that could have played a role in developing GWI. Most investigators agree that a combination of factors such as vaccinations, infectious diseases, biological weapons, chemical weapons, chemical agents (pyridostigmine bromide), oil well fire smoke, and depleted uranium (EIR, 2015) (Jansen, Mous, White, Posthuma, & Polderman, 2015) (EIR, 2015) could have led to the development of GWI.

Pyridostigmine Bromide (PB), commonly referred to as the “Nerve Gas Pill”, was considered a safe chemical agent that was given to troops to protect them against chemical warfare agents (Blokland, de Zubizaray, McMahon, & Wright, 2012) (EIR, 2015). Evidence suggests that GWI can be attributed in part by exposure to organophosphate (OPs) and carbamate acetylcholinesterase inhibitors (AChEis), including pyridostigmine bromide (PB), pesticides, and nerve agents. The enzymes known to be involved are paraoxonase (PON) for OPs and butyrylcholinesterase (BChE) for PB (Golomb, 2008).

A study of the Butyrylcholinesterase (BChE) genotype as a biomarker focused on genetic variants of the enzyme to find out whether the variants differ in their capacity for metabolizing AChE-inhibiting chemicals. The study found a subgroup of Gulf War veterans with a less common, BChE genotype. The association with GWI was significantly greater for this, less common BChE genotype compared to veterans with the common BChE genotypes. The study results provide preliminary evidence of gene-exposure interaction in relation to GWI (Steele et al. , 2015).

PB has also been shown to cause neuronal damage from reactive oxygen species (ROS). “ROS have been associated with IgM mediated autoimmune responses against ROS induced neoepitopes in depressed patients and this may also apply to CFS (chronic fatigue syndrome)” (Moss, 2012). One possibility is that ROS modified native molecules, triggering the autoimmune conditions leading to GWI (Moss, 2012)

Despite extensive research, the pathophysiology of GWI remains unknown. GWI, like several other similar syndromes, including fibromyalgia, CFS, and irritable bowel syndrome (IBS), all lack a characteristic biomarker (Institute of Medicine, 2013). Investigators have theorized that common complaints of joint pain, fatigue, and skin rash share a common immunological imbalance (Everson et al, 1999; O’Bryan et al., 2003). Many studies have reported immune abnormality among patients with CFS and fibromyalgia compared to controls (O’Bryan et al., 2003). GWI veterans showed an altered immune pathway activity following exercise challenges (Broderick et al., 2013). The overlap of GWI symptoms with of autoimmune symptoms, along with evidence of abnormal immune activation following exercise in GWI suggests an abnormal immune process in GWI (Broderick et al., 2013; Moss, 2012). The probable involvement of an abnormal immune process and the lack of a characteristic biomarker for GWI prompted our investigation of Human Leukocyte Antigen (HLA) in GWI, since HLA is associated with a number of autoimmune and rheumatological disorders (Gough & Simmonds, 2007). In a previous study, HLA was examined to see if it plays a role in genetic susceptibility to GWI by O’Bryan et al. (2003) without any statistically significant findings.

HLA Overview

HLA was discovered in the late 1950s and has become one of the most widely studied regions of the human genome. Initial interest in HLA was because of its application to organ transplantation (Mehra, 1989). However, studies in this locus have revealed associations with autoimmune, infectious, and inflammatory diseases (Fernando et al., 2008). Analysis of this region has been a challenge due to its highly polymorphic nature and extensive linkage disequilibrium (LD) (Fernando et al., 2008). However, because HLA exerts such a strong influence on the development of immunity, it has become a major theme in immunology.

The HLA locus, located in a 3500 kb segment of the major histocompatibility complex (MHC), is a collection of genes on a continuous stretch of DNA on chromosome 6 in humans. The MHC is divided into three groups: Class I, class II and class III. For MHC / HLA genes we focus on class I (HLA genes-A, -B, -C) and class II (HLA genes - DRB1, -DQA1, and -DQB1). Class III genes do not code for HLA but code for other genes related to inflammation and other immune responses.

Class I and class II have different cells that express them and different sources of antigens they present to T cells. Class I molecules, present on all nucleated cells, specialize in presenting intracellular proteins. When a cell expresses foreign proteins, such as viral proteins, the foreign particles are presented to CD8+ T cells, which recognize and kill the cell expressing the intracellular antigens (Stranford, 2013). Class II molecules on the other hand, are expressed on antigen presenting cells (APC) and specialize in presenting antigens from extracellular sources, such as bacteria and

fungi, after they have been engulfed. Class II molecules present the exogenous antigen peptides to CD4+T cells, activating the CD4+ T cells to destroy extracellular invaders (Stranford, 2013).

HLA genes in the MHC are one of the most polymorphic genes in humans; that is, there are many alternative forms of each gene, or alleles (Mack et al., 2013). HLA genes lie so close together that their inheritance is linked, so individuals tend to inherit the alleles encoded by HLA genes as a set (known as linkage disequilibrium). This set of linked alleles, which lie on a single chromosome (homolog), is referred to as a haplotype. Each individual inherits two sets of alleles or one haplotype from the mother and one haplotype from the father. HLA genes exhibit a codominant form of expression; both inherited maternal and paternal genes (haplotypes) are expressed at the same time and in the same cells (Stranford, 2013).

HLA nomenclature

HLA nomenclature is standardized and regulated by the *World Health Organization Nomenclature Committee for Factors of the HLA System*. HLA nomenclature starts with the gene name (e.g., A, B, DRB1, etc.) and has sets of digits trailing the gene name separated by colons. The first set of digits corresponds to the allele-family level while the second set of digits corresponds to the specific protein encoded. For example A*01: 101 is found on gene A, its allele is 01 and its protein code is 101. Two-digit resolution, also referred to as allele-family level, groups HLA based on alleles and is not protein specific. Four-digit resolution is commonly referred to as high-

resolution since it identifies the exact protein product of the gene. Optional suffixes may be added to an allele to indicate its expression status. For example, alleles that have not been found to be expressed have been given the suffix 'N' for null expression.

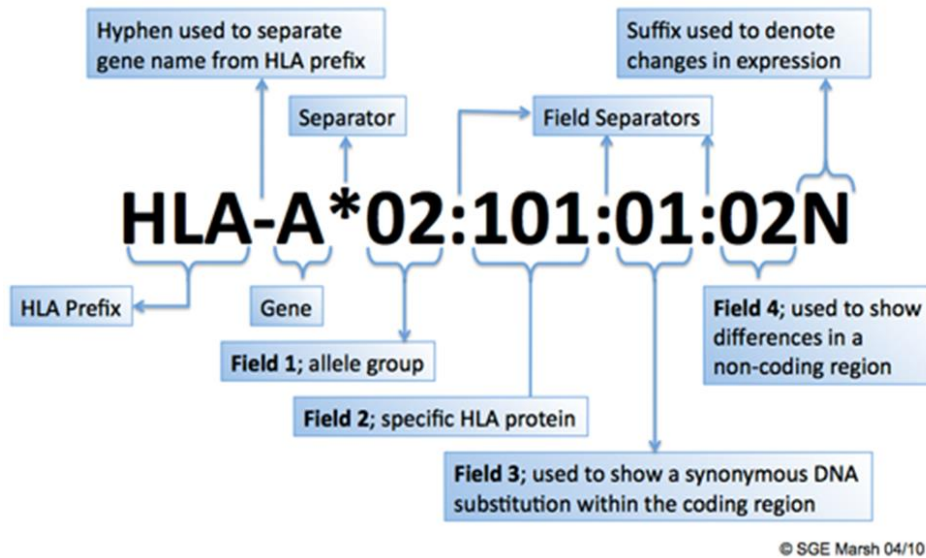


Figure 1

HLA and Other Diseases

HLA has been associated with many autoimmune diseases such as, type 1 diabetes (T1D), multiple sclerosis (MS), rheumatoid arthritis (RA), Graves' disease (GD), ankylosing spondylitis (AS) and systemic lupus erythematosus (SLE) (Gough, 2007). However, the molecular mechanisms underlying the disease association with the particular HLA molecule(s) are poorly understood. This is primarily due to (a) the high LD within the HLA region, (b) the fact that most HLA-associated diseases are multifactorial polygenic diseases in which disease susceptibility involves HLA allele(s), in combination with other genetic variants and environmental factors, and (c) the fact

that, in most cases, disease-relevant autoantigen(s) and mechanisms are largely unknown. Therefore, relating autoimmune disease associations with HLA alleles has remained largely hypothetical (Caillat-Zucman, 2009).

HLA Class I Disease Mechanisms

There are two commonly suggested hypotheses concerning disease mechanisms involving HLA class I molecules. These molecules play a role in presenting endogenous antigens, which may trigger a strong autoimmune response (a) through molecular mimicry (e.g., when microbial antigens are similar to self-antigens that activate autoreactive T-cells and trigger autoimmunity), and (b) by acting as super antigens (e.g., when antigens produce a strong non-specific immune response that then cross reacts attacking other tissues in the body). Viruses, for example, can alter HLA class I and II expression, potentially leading to greater antigen presentation to CD8+ T cells, with certain alleles more prone to viral/bacteria manipulation. Specific HLA variants and disease-associated mechanisms were identified for celiac disease (CD) and type 1 diabetes (T1D) (Caillat-Zucman, 2009). In other diseases such as ankylosing spondylitis (AS) and narcolepsy HLA associations are strong, HLA B*27 and DQB1*06:02 respectively, but the mechanisms underlying the disease associations are still unknown (Caillat-Zucman, 2009).

HLA Class II Disease Mechanisms

The HLA class II disease mechanisms suggest potential pathways that the HLA class II molecules could be involved in autoimmune diseases. For example differences in binding grooves of HLA molecules may allow autoreactive T-cells to escape tolerance and enter the periphery. This could cause incomplete thymic tolerance and can lead to the creation of a cell population that does not recognize all self-molecules. Polymorphic residues of the T-cell receptor (TCR) of HLA molecule could select autoreactive T-cells rather than a good T-regulatory population. The protective effects seen with specific HLA molecules may be due to the presence of T-regulatory cells that are able to keep autoreactive T-cells at bay, suggesting that diseases could be associated with a failure to protect rather than a predisposition to disease. These hypotheses suggest a series of potential mechanistic pathways by which the HLA class II molecules could be involved in disease onset by altering the T-helper and T-regulatory cell repertoire, or through changes in how the antigen is recognized in the periphery (Caillat-Zucman, 2009).

Research Goal

GWV remains a multi-symptom disease with unclear etiology and studies pointing to a genetic autoimmune component. The purpose of studying the HLA genotype of GWV veterans is to establish an immunological component of the disease and to explore the hypothesis that HLA is a contributing factor to GWV.

Study Design

The study was designed to analyze the relations between the HLA genotype of 82 GW era veterans (66 GWV and 16 controls) and presence and severity of GWV. Demographic information on participants is given in Table 1 below.

Table 1 Demographic information

	GWV	GWV_Control
Men	64	15
Women	2	1
Total	66	16
Age Range	39 -76	43 – 71
Average Age	50.6 ± 7.9	54.9 ± 10.2

Materials and Methods

Gulf War Veteran Population [Disease and Control Population]

Veterans Affairs (VA) medical records were used to identify potential participants to use as the case population for the GWI group. In order to remove other contributing factors, veterans were excluded from the study if they have autoimmune diseases, central nervous system disorders (e.g. Parkinson's disease, traumatic brain injury, etc.), mental health disorders, or current drug dependence. Prior to participation in the study all participants provided written informed consent, and participants were compensated for their time. The study protocol was approved by the Institutional Review Board (IRB) at the Minneapolis VA Health Care System. Participants completed a symptom presence/severity questionnaire developed for use in Kansas Gulf War (KGW) veterans. The KGW questionnaire allows for determination of GWI status according to the Centre for Disease Control and Prevention (CDC) criteria or the Kansas GWI case definition. Participants meeting either set of Gulf War case definition criteria were included in the study. A total of 82 participants, 16 controls (15 men, 1 woman; age range 43-71) and 66 GWI (64 men, 2 women; age range 39-76) were studied.

HLA Genotyping

Blood from the 82 participants was drawn into EDTA tubes, using a commercially available kit (Archive Pure cat. 2300730) from 5Prime (distributed by Fisher Scientific and VWR). The purified samples were then sent to the HistoGenetics labs in New York for high-resolution HLA Sequence-based Typing (SBT). The Gold

standard/High Resolution level was chosen to resolve all ambiguities in the antigen recognition sites (ARS); that is exon 2 and 3 for Class I (A, B, C) and exon 2 for class II (DRB1, DRB345, DQB1, and DPB1). For more details on SBT and standard operating procedures for HistoGenetics please see www.histogenetics.com.

Data Analysis

Classification

Stepwise Linear Discriminant Analysis (LDA) was used to identify alleles that classified participants as control or GWI using the SPSS statistical package. The classification variable was the group membership (GWI or control) and predictor variables were the number of allele copies for each allele. The allele set was validated using the leave-one out method option in SPSS, and by performing LDA on 10,000 bootstrap samples. In order to address the issue of bias in the classification due to unequal sample sizes (GWI = 66, Control = 16), separate LDAs were performed with samples with sizes equal to the smallest sample (N = 16) and classification rates were averaged (Sanchez, 1974). Lastly, confidence intervals on the classification rate were calculated using a chi-square analysis. In addition, a receiver operating characteristic (ROC) analysis was carried out and its associated statistics calculated.

Allele Effect: Protective or Predisposing

In order to assess the effect (protective or predisposing) of the discriminating alleles from the classification result, further analyses were carried out. The frequencies of the alleles found relevant were compared between the two groups (GWI vs. control) using a paired t-test. The odds ratio was calculated for each allele, along with its natural log (ln), and tested against the null hypothesis (the average does not differ significantly from zero), using a one-sample t-test. (Note: In calculating the odds ratio if any cell had

a count of zero, .5 was added to all cells to avoid taking the logarithm of zero or dividing by zero).

Symptom Domains affected by HLA

In order to determine which symptom domain was most affected by the discriminating alleles found in the classification result, a multiple linear regression analysis was performed. The symptom severity was the dependent variable and the number of copies of the discriminating alleles was the independent variable.

Comparing allele frequencies to other databases

To compare the frequencies of the discriminating alleles found in the classification results to the frequencies found in the literature, the website “Allele*Frequencies in Worldwide Populations” (<http://www.allelefrequencies.net/>) was searched. Three databases were found from the United States, with Caucasian populations and the relevant alleles in the databases: (1) “USA San Francisco Caucasian” (SF; N = 220; Skibola et al., 2012); (2) “USA Minnesota Olmsted” (MN; N = 339; Ovsyannikova et al., 2005); and (3) “USA Caucasian pop 5” (source: Center for Disease Control, CDC; N = 268; Rossman et al., 2003).

Results

Classification

A total of 144 unique HLA alleles were found in the dataset of 82 participants. The stepwise LDA identified 6 alleles (Table 1) that correctly classified the 82 subjects with a rate of 84.1% (Table 2). The robustness of the 6 alleles was verified using the leave-one-out classification method which classified 79.1% of the participants correctly. The robustness was also verified using 10,000 bootstraps of two sample sizes (N = 100, N = 200) which yielded overall correct classification rates of 80.1% and 81.2%. Given the small number of women, stepwise LDA was performed for men only (N = 79), yielding a classification rate to 86.1% and the leave-one-out classification rate to 81.0%. Finally, in order to address the issue of bias in the classification due to unequal sample sizes (GWI = 66, Control = 16), separate LDAs were performed with 4 permuted samples with size equal to the smallest sample (N = 16) and classification rates were averaged (Sanchez, 1974). The overall average classification rate by applying this procedure was 82.3% (range: 78.1% - 85.9%, N = 10,000 permutations).

Table 2 Results Stepwise Linear Discriminant Analysis (LDA)

A. Stepwise statistics				
	Allele	Tolerance	F-to-remove	Wilks' Lambda
1	DQB1*02:02	0.934	12.942	0.749
2	DPB1*06:01	0.913	10.162	0.725
3	DRB1*13:02	0.872	11.446	0.736
4	DRB1*08:11	0.943	8.249	0.709
5	DRB1*01:01	0.904	5.329	0.684
6	DPB1*01:01	0.923	4.228	0.675

B. Canonical discriminant function statistics			
Eigenvalue	% of variance	Cumulative %	
0.565	100	100	
Wilks' Lambda	Chi-square	DF	Significance
0.639	34.5	6	0.000005

Table 3 Classification Results

A. Classification table				
		Predicted		Total
		GWJ	Control	
Observed	GWJ	56	10	66
	Control	3	13	16
Total		59	23	82

B. Analysis of the two-way classification table			
Test	Value	DF	Significance (2-sided)
Pearson Chi-Square (corrected for continuity)	27.9	1	$P = 1.29 \times 10^{-7}$
Fisher's Exact Test			$P = 9.25 \times 10^{-7}$

C. Mantel-Haenszel common odds ratio estimate			
Estimated odds ratio ($\hat{\omega}$)	$\ln(\hat{\omega})$	SE of $\ln(\hat{\omega})$	Asymptotic significance (2-sided)
24.267	3.189	0.727	$P = 0.000011$
95% lower bound: 5.840			
95% upper bound: 100.83			

D. Sensitivity, specificity, and binomial confidence intervals			
	Observed	95% Confidence Intervals	
		Lower bound	Upper bound
Sensitivity	0.848 (84.8%)	0.804	0.893
Specificity	0.812 (81.2%)	0.715	0.910

Alleles protective or predisposing

To determine which of the 6 alleles were protective or predisposing, the frequency of the alleles in each group was calculated and compared between the 2 groups; in addition, the odds ratio for each allele was also calculated (Table 4). It was found that all 6 allele frequencies were significantly lower for the GWI group, as compared to the control group ($t = -5.789$, $DF = 5$, $P = 0.002$, paired t-test). The odds ratio was <1 for all alleles, and significantly so as a whole ($\ln(\hat{\omega}) = -1.792 \pm 0.383$ (mean \pm SEM), $t = -4.671$, $DF = 5$, $P = 0.005$, one-sample t-test against the null hypothesis that mean $\ln(\hat{\omega}) = 0$). These findings indicate that the discriminating alleles above have, collectively, a protective effect for the control group, and therefore by extension, a lack of protection in the GWI group. In fact, plotting the overall symptom severity against the number of allele copies showed a very strong negative relation ($t = -4.372$, $DF = 80$, $P = 0.000037$, $R^2 = 0.193$), such that the more copies of an allele the less the overall symptom severity (Fig. 2).

Table 4 Frequency and Odds Ratio

A. Frequencies and odds ratios					
	Allele	GWI frequency	Control frequency	Estimated odds ratio ($\hat{\omega}$)	$\ln(\hat{\omega})$
1	DQB1*02:02	0	0.0625	0.046*	-3.078
2	DPB1*06:01	0.007576	0.0625	0.114	-2.167
3	DRB1*13:02	0.0379	0.125	0.276	-1.289
4	DRB1*08:11	0	0.0312	0.0792*	-2.535
5	DRB1*01:01	0.0682	0.1562	0.395	-0.927
6	DPB1*01:01	0.0303	0.0625	0.469	-0.758

B. Percent of participants per group and allele			
	Allele	GWI	Control
1	DQB1*02:02	0	12.5
2	DPB1*06:01	1.5	12.5
3	DRB1*13:02	7.6	25
4	DRB1*08:11	0	6.3
5	DRB1*01:01	13.6	18.8
6	DPB1*01:01	6.1	12.5

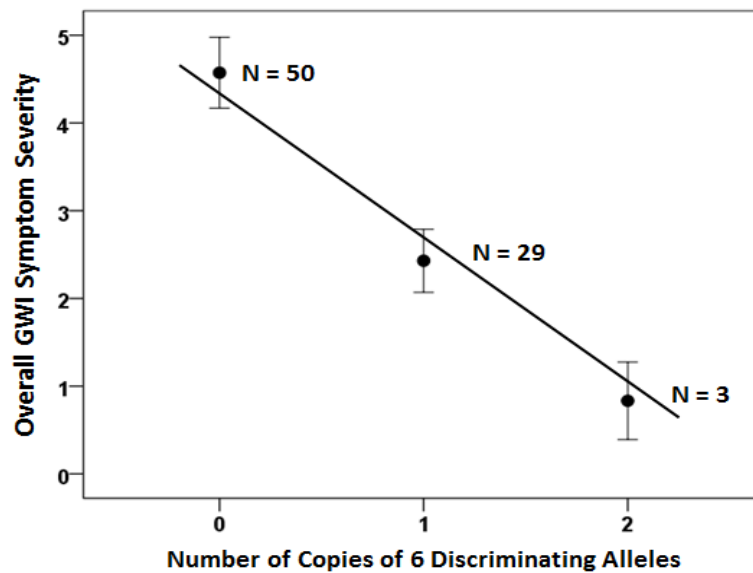


Figure 2

Symptom Domains affected by HLA

In order to determine which symptom was highly affected by the number of copies of 6 individual alleles, multiple linear regression analysis was performed on the severity of each symptom domain as the dependent variable. Statistically significant effects were found for Pain ($P = 0.01$, $R^2 = 0.199$), Fatigue ($P = 0.006$, $R^2 = 0.210$), and

Neurological/Cognitive/Mood ($P = 0.004$, $R^2 = 0.225$). All slopes of individual allele copies vs. symptom severity were negative in each model, indicating a consistent, robust effect across the domains. Finally, the regression analysis was not significant for Skin ($P = 0.911$), Gastrointestinal ($P = 0.576$), and Respiratory ($P = 0.598$) symptoms.

Comparing allele frequencies to other databases

In order to compare the 6 discriminating alleles to other databases in the literature, allelefrequencies.net was searched and allele frequencies were obtained for each one of three comparable databases (SF, MN, CDC). The mean frequencies (Fig. 3) of the 6 alleles were similar for the 3 databases, but the values for the GWI group were lower, whereas the values for the control group were higher. Next the $\ln(\hat{\omega})$ for each allele (6 total) was calculated between the database (SF, MN, CDC) and the control group. The $\ln(\hat{\omega})$ was also calculated for each allele (6 total) between the database (SF, MN, CDC) and the GWI group. Using a univariate ANOVA to assess the main effects, we found that the group (control or GWI) main effect was highly significant (Fig. 4; $P = 0.00019$), whereas neither the Database (SF, MN, CDC) main effect ($P = 0.911$; data not shown) nor the Database (SF, MN, CDC) x Group (control, GWI) interaction terms (Fig. 5; $P = 0.934$) were statistically significant (F-test in the ANOVA).

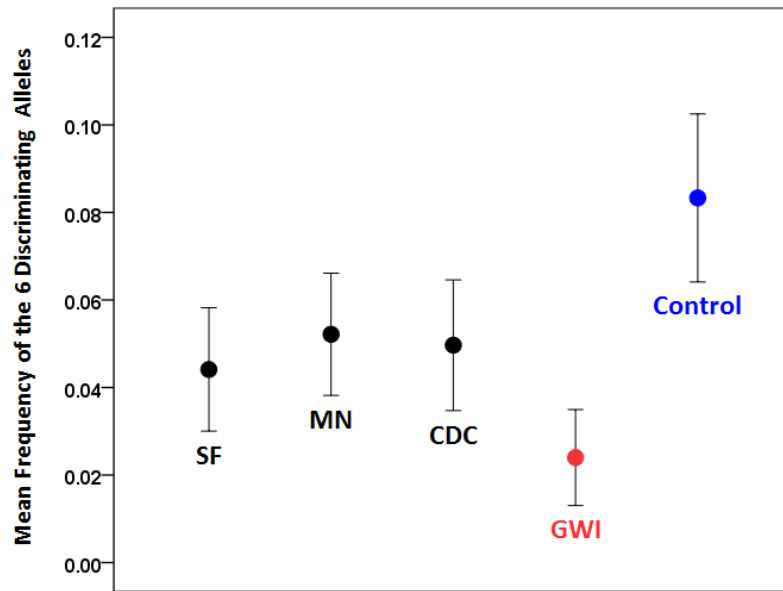


Figure 3

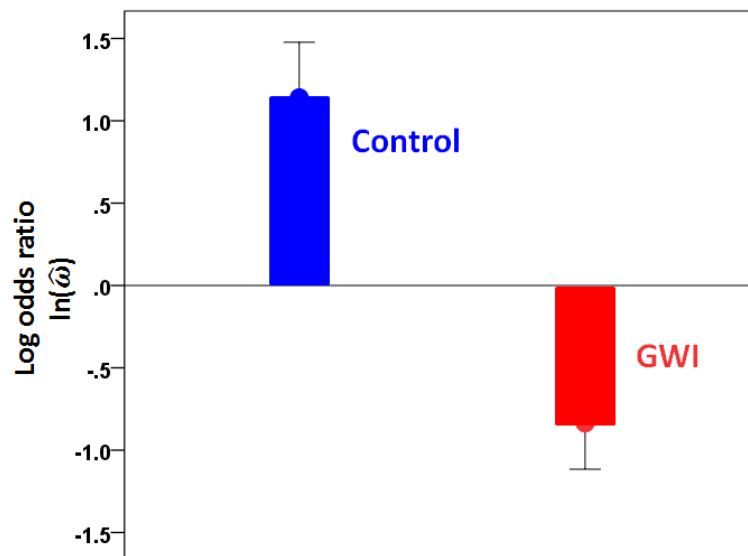


Figure 4

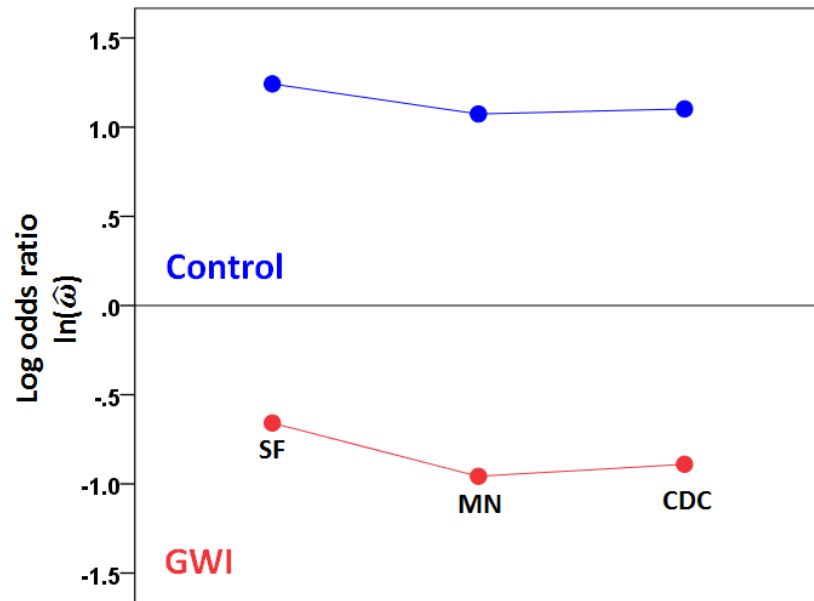


Figure 5

Discussion

The results from this study point to a HLA-based vulnerability (or lack of protection) in the GWI veterans and a reduced vulnerability (or additional protection) for the control group without GWI. Specifically, six class II HLA alleles: DQB1*02:02, DPB1*06:01, DRB1*13:02, DRB1*08:11, DRB1*01:01, and DPB1*01:01 were identified from the stepwise linear discriminant analysis to successfully classify the GWI veterans from the control group. Two alleles: DQB1*02:02 and DRB1*08:11 were absent from the GWI group, and the frequencies of the remaining four were all lower in the GWI group than in the GW control group (Table 4). Plotting the mean GWI symptom severity against the average number of copies of the six discriminating alleles (Fig. 2) shows a protective association of the alleles, a consistent result with the regression analysis.

In addition, in order to see which symptom domains the protective alleles relate to, multiple linear regression analysis was performed separately for each symptom domain. This analysis further differentiated the effect among specific symptom domains, showing highly significant overall protective effects for Pain, Fatigue, Neurological domains. The effect was not significant for the Skin, Gastrointestinal and Respiratory domains.

Comparison of the 6 allele frequencies to published databases (Ovsyannikova et al., 2005; Rossmann et al., 2003; Skibola et al., 2012) revealed that the GWI group had overall lower allele frequencies, and the control group had overall higher allele frequencies (Figs. 3-5).

The 6 protective alleles found in the LDA are all from HLA class II genes, which are involved in antibody production. Evidence has been shown that the HLA region is associated with decreased or increased antibody response (e.g. vaccines) in different individuals; for example, it has been suggested that HLA class II alleles are associated with antibody response to hepatitis B vaccine (Li, Nie, Li, & Zhuang, 2013). HLA class II associations with both high and low measles antibody responses have been described, both for measles vaccine given alone combined with other vaccines (Blackwell, 2009).

GW veterans were administered various vaccines (Institute of Medicine, 2000; L Steele, 2000) and were exposed to various chemical agents (Institute of Medicine, 2000; Steele et al., 2015) during the war. A number of deployed GW veterans developed GWI; however, GWI is also present in non-deployed or minimally exposed veterans (Steele, 2000). Thus, we hypothesize that vaccinations and chemical exposures of GW era veterans served as environmental triggers (“hits”) that contributed to developing GWI in genetically (HLA) “vulnerable” veterans. Specifically, certain HLA alleles in higher frequencies conferred protection, whereas their relative scarcity conferred vulnerability. It should be noted that both vaccinations (Israeli, 2012) and chemical exposures (Moss, 2013) have been implicated previously as contributing factors for the development of GWI. The results this study adds a genetic framework from which the effects of GWI can be interpreted and investigated for future work.

A major limitation of our study is the small number of participants studied which, nevertheless, yielded systematic, significant and robust differences between the control and GWI groups but also between those two groups and published HLA allele databases. In this context, it is especially noteworthy that high correct classification rates were

obtained in our extensive bootstrap analyses and permutation analyses (Sanchez, 1974) which documented the robustness of our results, i.e. their extension to larger sample populations. However, extensive bootstraps cannot substitute for validation in new, independent samples, which remains to be done. Finally, it is reasonable to assume that the six alleles identified in the present study are only part of a protective HLA makeup. We anticipate that a similar but large scale study will identify more such alleles and will provide a firm background to investigate the molecular mechanisms by which such protection/vulnerability may be mediated (Wahren-Herlenius & Dörner, 2013).

Bibliography

- Blanck, R. R. (1995). Unexplained Illnesses Among Desert Storm Veterans. *Archives of Internal Medicine*, 155(3), 262.
<http://doi.org/10.1001/archinte.1995.00430030050005>
- Blokland, G. A. M., de Zubicaray, G. I., McMahon, K. L., & Wright, M. J. (2012). Genetic and environmental influences on neuroimaging phenotypes: a meta-analytical perspective on twin imaging studies. *Twin Research and Human Genetics : The Official Journal of the International Society for Twin Studies*, 15(3), 351–71. <http://doi.org/10.1017/thg.2012.11>
- Broderick, G., Ben-Hamo, R., Vashishtha, S., Efroni, S., Nathanson, L., Barnes, Z., ... Klimas, N. (2013). Altered immune pathway activity under exercise challenge in Gulf War Illness: an exploratory analysis. *Brain, Behavior, and Immunity*, 28, 159–69. <http://doi.org/10.1016/j.bbi.2012.11.007>
- Caillat-Zucman, S. (2009). Molecular mechanisms of HLA association with autoimmune diseases. *Tissue Antigens*, 73(1), 1–8. <http://doi.org/10.1111/j.1399-0039.2008.01167.x>
- Everson, M. P., Kotler, S., & Blackburn, W. D. (1999). Stress and immune dysfunction in Gulf War veterans. *Annals of the New York Academy of Sciences*, 876, 413–8. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10415636>
- Fernando, M. M. A., Stevens, C. R., Walsh, E. C., De Jager, P. L., Goyette, P., Plenge, R. M., ... Rioux, J. D. (2008). Defining the role of the MHC in autoimmunity: a review and pooled analysis. *PLoS Genetics*, 4(4), e1000024. <http://doi.org/10.1371/journal.pgen.1000024>
- Fukuda, K., Nisenbaum, R., Stewart, G., Thompson, W. W., Robin, L., Washko, R. M., ... Reeves, W. C. (1998). Chronic multisymptom illness affecting Air Force veterans of the Gulf War. *JAMA*, 280(11), 981–8. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9749480>
- Golomb, B. A. (2008). Acetylcholinesterase inhibitors and Gulf War illnesses. *Proceedings of the National Academy of Sciences of the United States of America*, 105(11), 4295–300. <http://doi.org/10.1073/pnas.0711986105>
- Gough, S. C. L., & Simmonds, M. J. (2007). The HLA Region and Autoimmune Disease: Associations and Mechanisms of Action. *Current Genomics*, 8(7), 453–65. <http://doi.org/10.2174/138920207783591690>

- Gulf War Syndrome | Overview, Symptoms & Possible Causes. (2015). Retrieved October 16, 2015, from <http://www.ei-resource.org/illness-information/environmental-illnesses/gulf-war-syndrome/>
- Institute of Medicine National Research Council, 2000. (2000). Gulf War and Health. *Institute of Medicine National Research Council Gulf War and Health: Volume 1. Depleted Uranium, Pyridostigmine Bromide, Sarin, and Vaccines*. <http://doi.org/10.17226/9953>
- Institute of Medicine National Research Council, 2010. (2010). Gulf War and Health. *Institute of Medicine National Research Council Gulf War and Health: Volume 8: Update of Health Effects of Serving in the Gulf War*. <http://doi.org/10.17226/12835>
- Israeli, E. (2012). Gulf War Syndrome as a part of the autoimmune (autoinflammatory) syndrome induced by adjuvant (ASIA). *Lupus*, *21*(2), 190–194. <http://doi.org/10.1177/0961203311429552>
- Jansen, A. G., Mous, S. E., White, T., Posthuma, D., & Polderman, T. J. C. (2015). What Twin Studies Tell Us About the Heritability of Brain Development, Morphology, and Function: A Review. *Neuropsychology Review*, *25*(1), 27–46. <http://doi.org/10.1007/s11065-015-9278-9>
- Li, Z.-K., Nie, J.-J., Li, J., & Zhuang, H. (2013). The effect of HLA on immunological response to hepatitis B vaccine in healthy people: A meta-analysis. *Vaccine*, *31*(40), 4355–4361. <http://doi.org/10.1016/j.vaccine.2013.06.108>
- Mack, S. J., Cano, P., Hollenbach, J. A., He, J., Hurley, C. K., Middleton, D., ... Fernandez-Vina, M. (2013). Common and well-documented HLA alleles: 2012 update to the CWD catalogue. *Tissue Antigens*. <http://doi.org/10.1111/tan.12093>
- Medicine, I. of. (2013). *Gulf War and Health*. Washington, D.C.: National Academies Press. <http://doi.org/10.17226/13539>
- Mehra, N. K., & Taneja, V. HLA, immune response and disease. *Indian Journal of Pediatrics*, *56*(2), 171–9. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/2509344>
- Moss, J. I. (2012). Gulf War illnesses are autoimmune illnesses caused by reactive oxygen species which were caused by nerve agent prophylaxis. *Medical Hypotheses*, *79*(2), 283–4. <http://doi.org/10.1016/j.mehy.2012.04.043>
- Moss, J. I. (2013). Gulf War illnesses are autoimmune illnesses caused by increased activity of the p38/MAPK pathway in CD4+ immune system cells, which was

- caused by nerve agent prophylaxis and adrenergic load. *Medical Hypotheses*, 81, 1002–1003. <http://doi.org/10.1016/j.mehy.2013.09.014>
- O'Bryan, T. A., Romano, P. J., & Zangwill, B. C. (2003a). Human leukocyte antigens in Gulf War veterans with chronic unexplained multiple symptoms. *Military Medicine*.
- O'Bryan, T. A., Romano, P. J., & Zangwill, B. C. (2003b). Human leukocyte antigens in Gulf War veterans with chronic unexplained multiple symptoms. *Military Medicine*, 168(12), 1015–8. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/14719628>
- Ovsyannikova, I. G., Jacobson, R. M., Ryan, J. E., Vierkant, R. A., Pankratz, V. S., Jacobsen, S. J., & Poland, G. A. (2005). HLA class II alleles and measles virus-specific cytokine immune response following two doses of measles vaccine. *Immunogenetics*, 56(11), 798–807. <http://doi.org/10.1007/s00251-004-0756-0>
- Rossmann, M. D., Thompson, B., Frederick, M., Maliarik, M., Iannuzzi, M. C., Rybicki, B. A., ... Monos, D. (2003). HLA-DRB1*1101: A Significant Risk Factor for Sarcoidosis in Blacks and Whites. *The American Journal of Human Genetics*, 73(4), 720–735. <http://doi.org/10.1086/378097>
- Skibola, C. F., Akers, N. K., Conde, L., Ladner, M., Hawbecker, S. K., Cohen, F., ... Bracci, P. M. (2012). Multi-locus HLA class I and II allele and haplotype associations with follicular lymphoma. *Tissue Antigens*, 79(4), 279–286. <http://doi.org/10.1111/j.1399-0039.2012.01845.x>
- Steele, L. (2000). Prevalence and patterns of Gulf War illness in Kansas veterans: association of symptoms with characteristics of person, place, and time of military service. *American Journal of Epidemiology*, 152(10), 992–1002. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11092441>
- Steele, L., Lockridge, O., Gerkovich, M. M., Cook, M. R., & Sastre, A. (2015). Butyrylcholinesterase genotype and enzyme activity in relation to Gulf War illness: preliminary evidence of gene-exposure interaction from a case-control study of 1991 Gulf War veterans. *Environmental Health : A Global Access Science Source*, 14(1), 4. <http://doi.org/10.1186/1476-069X-14-4>
- Stranford., O. J. A. . J. K. J. P. and S. A. (2013). *Kuby Immunology. Journal of Chemical Information and Modeling* (7th ed., Vol. 53). Macmillan Higher Education, 2013. Print. <http://doi.org/10.1017/CBO9781107415324.004>
- Wahren-Herlenius, M., & Dörner, T. (2013). Immunopathogenic mechanisms of systemic autoimmune disease. *The Lancet*, 382(9894), 819–831. [http://doi.org/10.1016/S0140-6736\(13\)60954-X](http://doi.org/10.1016/S0140-6736(13)60954-X)