Commentary   OMICS International

Pesticide-induced Immunotoxicity May Underlie Synergistic Gene-environment Interactions that Increase Parkinson's Disease Risk for High-risk Genotype Individuals at rs3129882 in the *HLA-DRA* Gene

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Abstract

The Human Leukocyte Antigen (HLA) gene loci contains immune system-related genes involved in antigen presentation and in recent years, certain genetic variants in HLA genes have been associated with increased risk for late-onset Parkinson's disease (PD), the second most common neurodegenerative disorder, in combination with pyrethroid pesticide exposure. The mechanisms behind this interaction are currently under investigation. Evidence that immune responses may confer and/or modulate risk and progression of PD is mounting. Therefore, a clear understanding of how the immune system plays a role in this and other neurodegenerative disorders will be critical for successful development of disease biomarkers, and therapeutic interventions to delay or ameliorate the course of the disease.
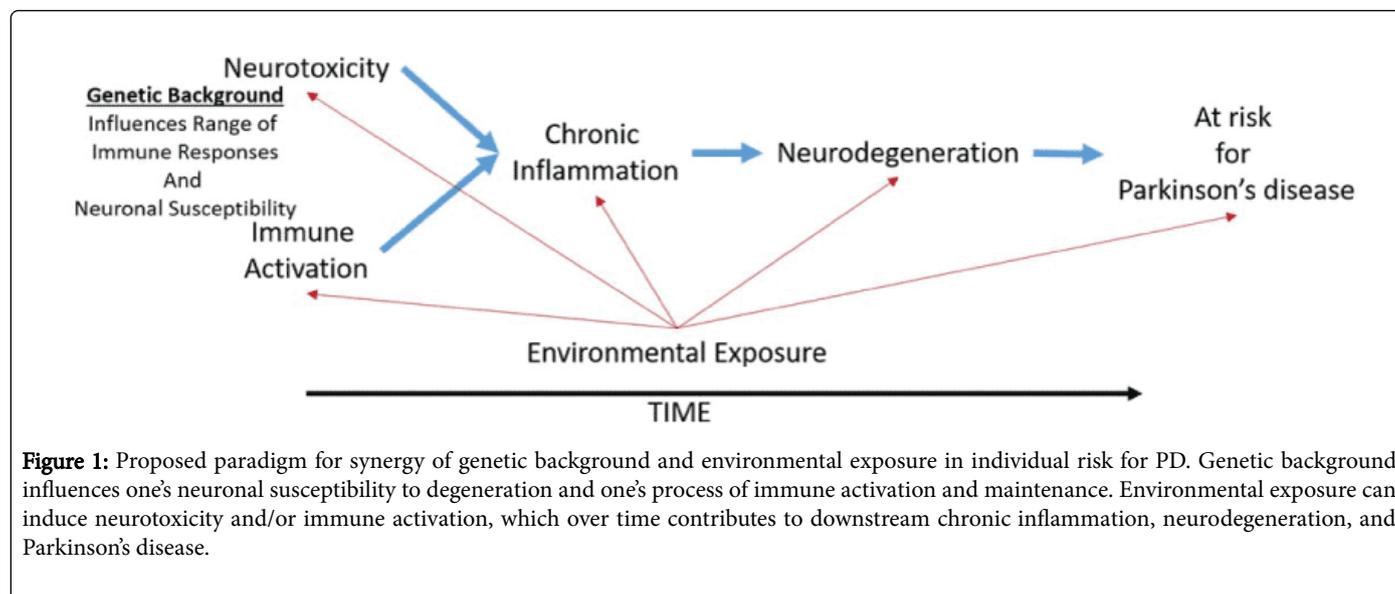
Introduction

Parkinson's disease (PD) is an age-related chronic, progressive neurodegenerative disorder that is defined clinically as a set of motor symptoms (bradykinesia, rigidity, postural instability, and resting tremor); histopathologically, PD is characterized by the degeneration of specific neuronal populations associated with α-synuclein inclusions called Lewy bodies [1,2]. The precise etiology of late-onset PD remains unknown, hence the term idiopathic PD [3,4]. Multiple mechanisms including mitochondrial damage, oxidative stress, proteinopathy, and inflammation have been implicated in the neuronal injury and loss that occurs in PD [1,5,6]. The relative importance and relevance of these different mechanisms continues to be enthusiastically debated. The activation of the immune system has been shown in numerous ways in the pathophysiology of PD [7-10]. Activated microglia can be visualized on PET scan and on post-mortem examination in brain regions affected in PD, including the pons, midbrain, thalamus and cortex [8,11,12]. Infiltration of T lymphocytes has also been demonstrated in the human PD brain, suggesting that activation of the adaptive immune system occurs during its development [13]. These findings as well as alteration of peripheral immune cell populations and cytokine levels strongly support that a dysregulated inflammatory state occurs in a significant fraction of PD patients [14-16]. It is unclear, however, whether this dysregulated inflammation is the cause or by-product of neuronal dysfunction and death. The normal function of the immune system is to discriminate self from non-self while specifically targeting and eliminating potentially pathogenic or toxic antigens. As novel genetic and environmental risk factors for PD are elucidated, it logically follows that the immune system provides a mechanistic link between one's genetic background and one's environmental exposures which come together to determine our overall risk for development of the disease. This idea is particularly attractive as many of the proteins most highly associated with PD pathogenesis, including alpha-synuclein and LRRK2, are enriched in both immune cells and neuronal populations [17].

Linking Genetic and Environmental Risk Factors for Parkinson's Disease through HLA

The identification of the common genetic variant or single nucleotide polymorphism (SNP) at *rs3129882* within the non-coding region of the *HLA-DRA* gene in genome-wide association studies (GWAS) provided a testable mechanism of disease susceptibility unique to the immune system [18-21]. Antigen presentation is uniquely responsible for activating the adaptive immune system through presentation of peptides on Human Leukocyte Antigen (HLA) proteins [22-24]. Once the adaptive immune system, T and B lymphocytes, has been directed against antigens presented on HLA proteins, it can orchestrate inflammatory responses as well as targeted destruction of specific cells. In other words, an adaptive immune response could selectively target the neuronal populations that degenerate in PD and help drive the pathogenic process underlying disease. Therefore, although it has been proposed that the selective vulnerability of dopaminergic neurons to degeneration stems from their inherent vulnerability to oxidative stress, mitochondrial dysfunction, protein aggregation, and inflammatory stress, it is also possible that selective targeting for destruction by the adaptive immune system may represent an additional mechanism that contributes to neuronal loss. In fact, this idea may also help explain the involvement of other non-dopaminergic neuronal populations in PD which are not thought of as "vulnerable" to cellular stressors and the loss of which gives rise to non-motor symptoms of PD (Figure 1).

Figure 1: Proposed paradigm for synergy of genetic background and environmental exposure in individual risk for PD. Genetic background influences one's neuronal susceptibility to degeneration and one's process of immune activation and maintenance. Environmental exposure can induce neurotoxicity and/or immune activation, which over time contributes to downstream chronic inflammation, neurodegeneration, and Parkinson's disease.

GWAS reported that the G allele at *rs3129882* SNP in the *HLA-DRA* gene was present at a higher frequency in subjects with PD of European ancestry [19]. In individuals of European ancestry, homozygous carriers of the high-risk G allele had a 1.7-fold increased risk of developing PD in one of the largest GWAS performed including this locus [19]. We then set out to determine if we could go beyond the GWAS association and determine whether individuals of European descent with GG versus AA genotype at *rs3129882* had antigen presenting cells with altered expression of HLA mRNA and protein [25]. We found that GG individuals displayed 1) increased baseline expression of HLA-DR on the major antigen presenting cell (APCs) populations peripherally (B cells and monocytes); and *GG* individuals with PD displayed 2) increased baseline expression of MHC-II mRNA in APCs from subjects with the high-risk GG genotype; 3) greater inducibility of HLA-DQ surface expression in monocytes compared to AA (low-risk) individuals with or without PD; and 4) greater inducibility of MHC-II mRNA expression with IFN-γ stimulation in cells from PD patients with the high-risk GG genotype [25]. In general, the high-risk SNP seems to be associated with higher levels of MHC-II expression [25]. Higher levels of MHC-II expression during an immune response could allow for activation of more numerous and diverse epitopes. These epitopes could then enhance or initiate a neuroinflammatory response that can hasten neurodegeneration. Antigens which could trigger neuroinflammatory responses in PD are yet to be definitively identified but studies show that post-translationally modified alpha-synuclein or dopaminergic neuronal proteins may be targets [26-29].

Given the functional aspects we observed on the associations with the genetic risk factor and surface-expression of MHC-II proteins on APCs, we sought to determine if there were any environmental exposures that synergized with the *rs3129882* SNP. Indeed, we demonstrated that chronic exposure to pyrethroids in people with the high-risk *GG* genotype significantly increased risk of PD [25]. Pyrethroids are known to exert neurotoxic effects in insect brain cells by inhibiting voltage-gated sodium channels [30]. Interestingly, overt neurotoxicity is not often observable in humans in part due to the diversity of channels proteins present on human neurons [31]. However, pyrethroids can cause hyperexcitability in neurons via

reactive oxygen species and have been documented to exert dopaminergic dysfunction in rodents [30,32-37]. Although pyrethroids were deemed to be safe for humans because their brain penetrance was minimal, these same sodium channels are present on peripheral immune cells and their function in these cell types is not well understood [38]. Pyrethroids can be absorbed through the respiratory tract or skin with a plasma half-life in humans of about 6-7 hours [39]. In exposed farm workers, plasma levels of pyrethroid metabolites could reach up to 10 ng/mL [40]. These compounds are metabolized by esterases or cytochrome P450 enzymes in the liver and then excreted in the urine [41]. Pyrethroid exposure in humans has shown to be associated with alterations in peripheral immune cell populations as well as in serum cytokine levels [42-44]. Treatment of mammalian immune cells with pyrethroids resulted in alterations in cytokine secretion and production of reactive oxygen species [45-47]. A recent study has shown that pyrethroids can activate primary microglia in a sodium-channel dependent manner [38]. It is unknown how pyrethroids may affect the antigen presentation process. Pyrethroids may accentuate ongoing immune responses by acting on APCs such as microglia or macrophages and/or by acting on the T cells activated by APCs.

Contextualizing the Immune System in Gene-Environment Interactions

Indeed, our preliminary studies *in vitro* reveal that pyrethroids can accelerate the replication rate of Jurkat cells, a T-cell leukemia cell line, as measured by a flow cytometric cellular division dye. Two types of pyrethroids, esfenvalerate and permethrin, can both accelerate the number of divisions in this cell line over a 60-hour period (Figure 2). Rotenone, a mitochondrial inhibitor linked to increased PD risk, only accelerated cell replication at the lowest dose and significantly dampened it at higher doses. These data suggest that one of the ways in which pyrethroids could increase risk for PD is through immunomodulation at the T cell level at the very minimum. It remains to be seen whether pyrethroids can alter APC function and synergize with elevated HLA expression and inducibility observed in human immune cells from individuals with the high-risk *rs3129882* SNP.
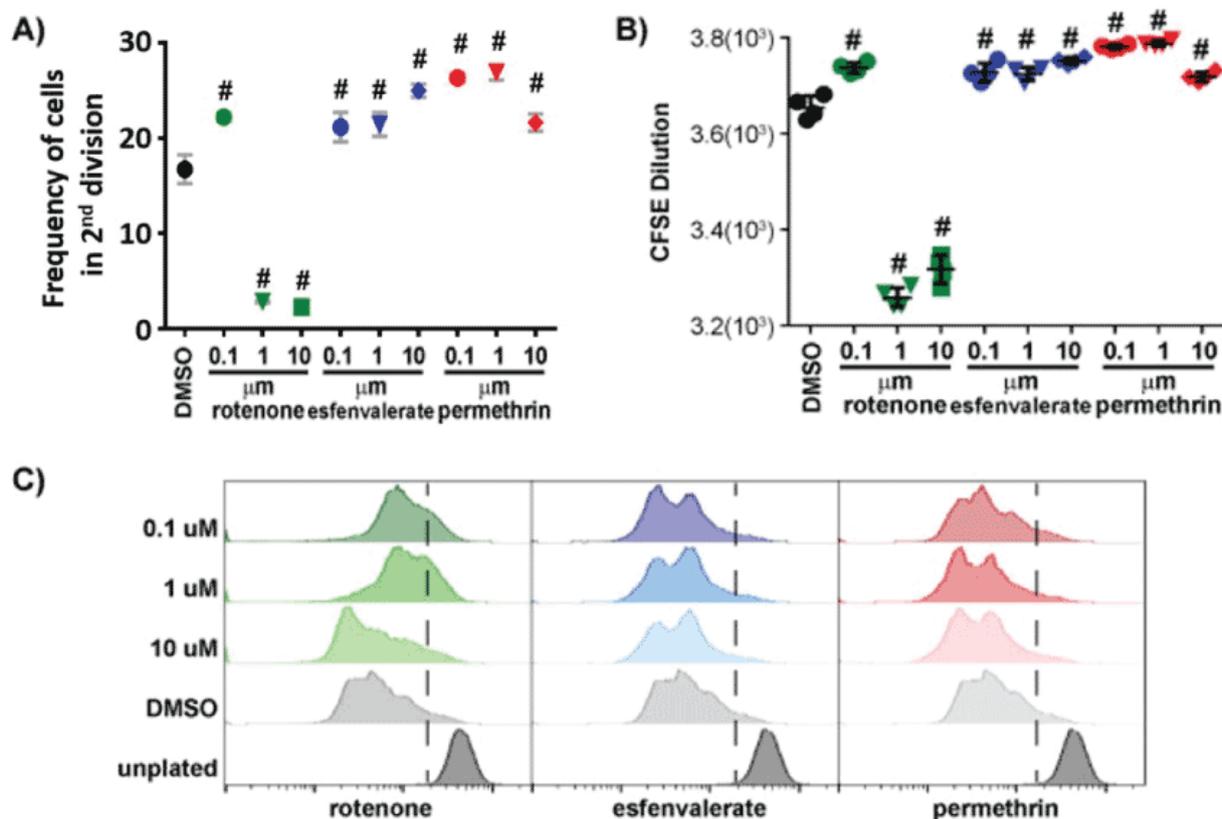
Figure 2: Pyrethroids increase rate of Jurkat T cell line proliferation. Carboxyfluorescein succinimdyl ester (CFSE)-labeled Jurkat T cells were allowed to proliferate in the presence of various concentrations of pyrethroids for 60 hrs and then were analyzed by flow cytometry in quadruplicate. Frequency of cells in 3rd division (A), change in median fluorescence intensity (MFI) of population CFSE dye (B), and representative flow cytometry plots (C) are indicated. One-way ANOVA with Sidak *post-hoc* test was used to assess significance with treatment and stimulation as covariates. ***p<0.001, **p<0.01, *p<0.05.

Besides pyrethroids, it is possible and even likely that other environmental factors may synergize with the *rs3129882* high-risk SNP to increase risk for PD. Other environmental factors independently associated with PD include traumatic brain injury, heavy metals, and viral infections [48-50]. In particular, certain strains of influenza are known to cause parkinsonism, such as the encephalitis lethargica epidemic that arose after the Spanish Flu of 1918 [51]. In animal models, infection with neurotropic influenza viruses can cause PD brain pathology [52]. From a small subset of the subjects in our study, we assessed whether antibody titers against the H1N1 flu virus differed among individuals with the high-risk versus low-risk *rs3129882* SNP. In our small sample, there was no significant difference in these titers regardless of whether individuals had the high-risk *GG* genotype or had PD (Figure 3). The strain of flu virus and time since infection would be particularly important determinants of any provoked neuroinflammatory response which may explain the negative results in this preliminary experiment. Larger studies which test a variety of flu strains from various years may be more revealing as to whether influenza infection can synergize with the *rs3129882* SNP. It remains to be determined whether influenza is a true etiologic factor for PD through the HLA locus.
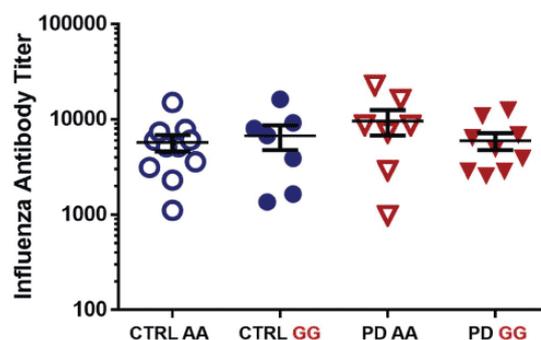

Figure 3: Antibody titers to influenza virus do not differ between individuals with Parkinson's disease and healthy controls or between those with the high-risk versus the low-risk genotype at the *rs3129882* SNP. Serum from subjects was assayed by ELISA for detection of the 2012 H1N1 flu virus. One-way ANOVA with Sidak *post-hoc* test was used to assess significance with treatment and stimulation as covariates. ***p<0.001, **p<0.01, *p<0.05.

As we move forward in understanding neuroinflammation in PD, it will be critical to identify the antigens that initiate and maintain immune responses that selectively target and destroy neurons. New studies implicate Parkin and PINK-1, two proteins associated with genetic and sporadic forms of PD, may play a role in the regulation of antigen presentation of mitochondrial antigens [53,54]. Nearly all of the proteins associated with PD, including alpha-synuclein and LRRK2, are present in both immune cells and neurons but their functions in immune cells are not clear and have been underexplored within the context of neuroinflammation in PD [55]. Our study also demonstrated that the *rs3129882* SNP association may be reversed or absent in specific ethnic groups suggesting a genetic or epigenetic mechanism linked to the *rs3129882* SNP in different ways different populations [25]. Identification of the underlying genetic or epigenetic mechanisms linked to the *rs3129882* SNP will be critical to our understanding of immune pathophysiology underlying PD development and/or progression. In summary, we posit that the immune system is likely to be the nexus for the gene-environment interactions that contribute to immunotoxicity and development of PD. Understanding the role of immunity and immune dysfunction in PD will help us delineate the etiology and intervene in the pathophysiology of this debilitating disease.

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