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The Role of Immune Mechanisms in Aging and Neurodegeneration

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The Role of Immune Mechanisms in Neurodegeneration and Aging

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ABSTRACT

The Role of Immune Mechanisms in Aging and Neurodegeneration

By George T. Kannarkat

In recent years, it has become increasingly clear that inflammation is a key driver of neurodegenerative pathology that can synergize with other factors such as aging and neuronal dysfunction. Two particularly interesting pathways in the immune system conferring disease risk are regulation of G-protein signaling (RGS) and antigen presentation. Specifically, the RGS10 protein is a negative regulator through its ability to accelerate deactivation of $G\alpha_i$ or $G\alpha_q$ molecules and it has been implicated in age-related macular degeneration. The loss of RGS10 in a mouse leads to increased dopaminergic neuronal vulnerability to inflammation and dysregulated immune responses. In this work, it was demonstrated that there are alterations in immune cell populations but not dopaminergic neuron homeostasis with age in RGS10-/- mice. Furthermore, it was shown that loss of RGS10 alters immune cell chemotaxis. Because of these roles for RGS10, it could prove to be an interesting target for therapy in treating neurodegenerative disease.

Antigen presentation has been implicated as a risk factor for Parkinson's disease through many genome-wide association studies identifying polymorphisms in the Major Histocompatibility Complex (MHC)-II locus. Herein, it was determined that a polymorphism in the *HLA-DRA* gene conferring higher risk for Parkinson's disease was associated with increased expression of MHC-II molecules as well as inducibility of these molecules in response to IFN-γ. Furthermore, it was found that people with the high risk polymorphism who were exposed to the commonly used class of insecticides, pyrethroids, were at increased risk for Parkinson's disease compared to the low risk

polymorphism demonstrating a gene-environment interaction between pyrethroids and the MHC-II locus. This class of insecticides were shown to have immunomodulatory properties suggesting that pyrethroids synergize with antigen presentation to dysregulate immune responses that put people at risk for neurodegeneration.

Both regulation of G-protein signaling and antigen presentation have key roles in regulating immune responses that influence risk for neurodegenerative disease. The targeting of these processes for treatment of neurodegeneration has not been explored. It will be critical to understand and describe the role of the immune system in promoting and complementing other pathogenic processes intrinsic to neurons.

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Chapter 1: Introduction

Incorporates previously published work[1].

Aging is the greatest risk factor for neurodegeneration but how the interplay between the processes that leads to neurologic disease is not well-understood. The role of the immune system as an important driver of pathogenesis as we age is being increasingly recognized. In all fields of neurodegeneration, a great need exists for disease-modifying therapies, better comprehension of disease pathogenesis, and for identification of relevant biomarkers for early diagnosis and monitoring of disease progression[2]. Targeting the immune system through two interesting pathways of G-protein signaling and of antigen presentation could address these needs. Both of these pathways play critical roles in the immune system and herein, the importance of regulation of G-protein signaling in aging of the immune cells and their function as well as antigen presentation in increased risk for Parkinson's disease (PD) have been demonstrated.

1.1) RGS10 has an important role in aging of the immune system

1.1a) Function of RGS proteins

RGS proteins are GTPase accelerating proteins (GAPs) that function to negatively regulate the signaling that occurs via G-proteins[3]. Typically, a heterotrimeric G-protein is activated upon its α-subunit binding guanine triphosphate (GTP) allowing for separation of the subunits into G_{α}-GTP and $G_{\beta\gamma}$ to perform their effector functions in signaling pathways[4]. RGS proteins negatively regulate this process by promoting hydrolysis of the GTP bound to the G_{α} subunit, dampening downstream csignaling[4]. Another function of RGS proteins is to help assemble signaling complexes via interactions with other proteins through their non-RGS domains[3]. The RGS domain has GAP activity with the remaining domains determining its interaction with other proteins[3]. Most RGS proteins are non-selective for $G_{\alpha i}$ or $G_{\alpha q}$ subunits[3]. RGS proteins can be divided into four families based on homology and RGS10 is the smallest of these proteins[3]. These proteins exist in all

eukaryotic organisms indicating their fundamental role in cell signaling and function[3,5]. The ubiquitous nature of RGS proteins suggests that tight regulation of this system is necessary to promote normal homeostasis[6].

1.1b) RGS proteins have important roles in regulating immune cell function

G-protein coupled receptors are involved in regulating numerous immune cell functions including cellular adhesion and chemotaxis[7]. RGS proteins are known to regulate many functions critical to immune function[8]. All RGS proteins are expressed in immune cells with differing levels of RGS subtype depending on immune cell type and activation state[9]. After activation of immune cells, RGS proteins are highly selectively regulated depending on RGS subtype and cell type[8]. Differential regulation of RGS proteins has been implicated in B cell chemotaxis, signaling through lymphocyte antigen receptor, and response to IL-2 stimulation[10-12]. Chemokine receptors are seven-pass transmembrane proteins that are G-protein coupled receptors, providing a role for RGS proteins in immune cell migration[7]. Immune cell migration is critical for proper development and maintenance of immune cells as well as competent immune responses to infectious challenge[7]. For example, RGS1-/- mice demonstrated increasing homing of B cells to lymphoid organs and increased motility of these cells within lymphoid organs[4,13]. In addition, RGS2-/- mice have impaired T cell activation in response to antigen stimulation and reduced antiviral immunity[14]. Many other RGS proteins have been implicated in immune cell migration and cytokine secretion[15-17]. Given the importance of G-protein signaling in immune cell function, RGS proteins play an important role in regulating immunity.

1.1c) RGS proteins have a multifaceted role in aging

Aging is a process characterized by irreversible changes in cell types throughout the body that lead to increased risk for disease, loss of homeostatic control, and increased

likelihood of death[18]. Mutation in G-proteins and associated proteins have been implicated in the development of cancers, particularly ones that respond to hormones[19,20]. Drosophila lacking their endogenous RGS protein had longer lifespan as well as increased resistance to oxidative stress via the protein kinase A pathway upstream of superoxide dismutase expression[21,22]. Furthermore, rat fibroblast cells with knockdown of RGS14 had increased resistance to oxidative stress[21,22]. In the brain, most expression of G-proteins and RGS proteins decreases with age. This change in expression is implicated with changes in production of neurotransmitter production that may be related to diseases such as Alzheimer's disease[23,24]. Many of these changes in G-protein signaling are related to alterations in composition of membrane lipids with aging, particularly in cardiovascular disorders, but the relationship between these alterations is not clearly understood[23]. RGS proteins and their activities change with age in multiple body systems. Further study is needed to identify the specific mechanisms in aging that RGS proteins regulate that contribute to disease.

1.1d) RGS10 regulates neuroimmune interactions

RGS10 is the smallest of the RGS family of proteins and is a selective activator of Gαi GTPase activity[25]. It is present in the cytoplasm, nucleus, and can be membrane-associated[26,27]. Phosphorylation and palimtoylation control localization to the nucleus and membranes, respectively[26,27]. For example, RGS10 palmitoylation has been implicated in regulation of inward rectifier potassium channels that requires association with the cell membrane[27]. The physiologic substrate(s) of RGS10 have not been identified but it has been implicated in modulating a variety of pathways in both immune cells and neurons. The expression and function of RGS10 in both neuronal and immune cells suggests that it is an important regulator of neuroinflammation[28-30]. In the rat brain, RGS10 expression is especially high in the dentate gyrus and superficial layers of the neocortex and dorsal raphe[31]. In mice and humans, RGS10 is expressed in brain, testis, lung, heart, lymphoid

organs, and bone marrow[32,33]. Between rodents and humans, there is a high degree of homology in the RGS10 protein[33]. RGS10 has been shown to regulate a diverse range of cellular pathways such as gonadotropin-releasing hormone receptor, chondrocyte differentiation, potassium current in myocytes, RANK-L induced differentiation of osteoclasts, chemoresistance in ovarian cancer, and serotonin and dopamine receptor signaling[34-40]. In a rat seizure model, RGS10 was the most highly induced RGS in the affected brain area[31]. In human disease, RGS10 has been implicated in risk for schizophrenia and age-related macular degeneration[41-43]. A missense mutation in the RGS domain of human RGS10 increases risk for schizophrenia[43]. Increased risk for age-related macular degeneration, characterized by chronic microgliosis in the retina, has been associated with a chromosomal locus that contains the RGS10 gene[41,42]. Aspirin-resistant platelets from people with metabolic syndrome, a condition associated with elevated systemic inflammation, have elevated levels of RGS10[44]. Reduction of RGS10 expression in a mouse model of periodontal disease demonstrated decreased inflammation and decreased bone loss[45]. Given these associations, it is clear that RGS10 is poised to regulate functions of both neuronal and immune cells that play roles in neuroinflammatory reactions.

Indeed, loss of RGS10 in a mouse has profound effects on neuroinflammation. On a mixed 129/C57BL/6 background, RGS10-/- animals have chronic microgliosis throughout the brain, an altered central nervous system (CNS) cytokine and chemokine milieu, and loss of dopaminergic neurons in response to peripheral lipopolysaccharide (LPS) administration[46]. LPS stimulation induces more punctate and increased nuclear staining of RGS10 in primary mouse microglia[46]. Interestingly, the loss of RGS10 leads to increased LPS-induced cytokine secretion from primary mouse microglia and a corresponding increase in vulnerability of primary dopaminergic neurons to inflammation[47]. The use of a 6-hydroxydopamine model demonstrated increased dopaminergic neuron susceptibility and increased neuroinflammation in the RGS10-/-

mice. Restoration of RGS10 to these RGS10-/- cells both *in vitro* and *in vivo* reverses these phenotypes indicating the specificity of these effects[47]. The phenotype of mouse peritoneal and bone-marrow derived macrophages is similar to the proinflammatory phenotype of microglia[30]. RGS10 has also been implicated in negative regulation of chemotaxis, similar to other RGS proteins, in T cells[28]. In neurons, it has been demonstrated that RGS10 provides protection against TNF-induced neurotoxicity through a phospho-cAMP response element binding pathway[29]. The physiological substrate and the most relevant biological pathways that RGS10 regulations in neurons and immune cells have not clearly been defined. It is also unclear how it plays a role in aging since that is the largest risk factor for neurodegenerative disease. Better understanding of these aspects of RGS10 function could potentially aid in the development of therapeutics that target this regulator of neuroinflammation.

1.2) Inflammation as a Driver for Pathogenesis of Parkinson's Disease

1.2a) Etiology and Pathogenesis of PD

Clinical diagnosis of PD is established through physical examination and rating of motor symptoms including bradykinesia, resting tremor, and muscle rigidity but it is now recognized that REM-behavior disorder, constipation, depression, and anosmia precede manifestation of motor symptoms by years to decades [48]. Hereditary forms of PD, caused by specific gene mutations, only comprise 5-10% of PD cases [49]. These genetic forms of PD typically have an earlier age of onset and provide insight into the potential role of ubiquitously expressed proteins in neuronal and immune dysfunction in sporadic PD [50]. Other non-motor symptoms develop later in disease progression such as psychosis, autonomic dysfunction, and cognitive abnormalities [49,51]. The progressive, multi-system pattern of PD symptomatology is consistent with a prominent role for a chronic

inflammatory process.

PD pathology is characterized by chronic neuroinflammation, Lewy body inclusions, and loss of dopamine-producing (DA) neurons in the substantia nigra parts compacta (SNpc) of the midbrain [49,51]. The Braak hypothesis (**Fig 1**) proposes a PD-specific pattern for progression of Lewy body pathology, intraneuronal aggregate of various proteins, including α-synuclein, tau, and ubiquitin. Lewy bodies are hypothesized to initially form in the olfactory bulb or the gastrointestinal tract (areas that are constantly exposed to the environment). As disease progresses, it is thought that pathological hallmarks then appear in the vagus or olfactory nerve and subsequently in the brainstem. When patients present clinically with motor symptoms, over 70% of DA neurons in the SNpc have died and significant Lewy body pathology as well as inflammation is present in that region. In final stages of the disease, pathology also occurs in the forebrain and cortical regions. An attractive feature of this hypothesis is that it may explain the presence and timing of non-motor and motor symptoms, as well as the multi-systemic nature of the disease that could be mediated in part by the spread of inflammation. Efforts to use non-motor symptom-based screening to identify people "at-risk" for PD are underway and will be important in clarifying underlying pathological mechanisms [52].

1.2b) Etiology of Sporadic Parkinson's Disease

The etiology of sporadic PD is undetermined because significant loss of DA neurons (~70%) has already occurred by the time of clinical presentation [53]. Nevertheless, it is generally accepted that both genetic and environmental factors contribute. Multiple genetic polymorphisms have been associated with sporadic PD through genome-wide association studies (GWAS) (**Table 1)** [54]. The results of these GWAS identify specific genetic loci that contribute to disease susceptibility. Such loci include genes for α-synuclein, glucocerebrosidase, and tau. Thus, genetic background combined with specific

environmental exposures could promote the manifestation of the pathologic process underlying PD.

Environmental exposures also modify the risk for PD. Indeed, the identification of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was critical in recognizing the loss of these neurons as the cause of motor symptoms in disease [55,56]. Upon absorption into the bloodstream, the highly lipophilic MPTP molecule quickly enters the CNS, where it is metabolized by monoamine oxidase-B into 1-methyl-4-phenylpridinium (MPP+). MPP+ inhibits cellular respiration by direct inhibition of the mitochondrial transport chain leading to neuronal injury and death [57]. Unlike MPTP, the mechanisms through which other environmental exposures modulate risk of PD are unclear. PD risk is increased by pesticide/organophosphate exposure and head trauma while it is decreased by moderate amounts of cigarette use and caffeine consumption [58-60]. Positive associations of PD with influenza, toxoplasmosis, and autoimmunity have been reported while non-steroidal anti-inflammatory drugs decrease risk for PD [61-69]. Genetic factors, as well as environmental factors clearly contribute to the development of PD; however, neither is believed to be independently sufficient to cause sporadic disease. Thus, it is generally accepted that the complex interplay between genetic and environmental factors predisposes people to the development of sporadic PD.

The immune system is in constant dialogue with our environment, tolerating certain exposures or antigens while responding to others. Dysregulated immune responses lead to disease. An inadequate response to an infection leads to bacteremia while hyper-reactivity to a harmless antigen can cause allergy. The innate immune system plays a key role in initiating inflammation in response to conserved antigen or cell death signals such as bacterial flagella, single-stranded viral DNA, or release of inflammatory cytokines from injured cells. The activated innate immune system will then induce an adaptive immune response, which are specific, targeted, and highly potent against the antigens present in the milieu of the inflamed region. Since adaptive immune

cells can promote both anti-inflammatory and pro-inflammatory reactions in response to specific antigens, they are uniquely suited to modulate inflammation in the context of certain environmental exposures and neuronal dysfunction [70,71]. In addition, complex immunoregulatory processes that require intricate crosstalk between the adaptive and innate immune system occur at mucosal surfaces that are constantly exposed to antigens [72]. Interestingly, the Braak hypothesis suggests that these mucosal sites (nasal and intestinal mucosa) may be where PD pathology is initiated. Thus, the immune system is poised to determine the body's response to various environmental exposures in predisposing to or protecting against PD. Furthermore, because PD is a multi-system progressive disease, the immune system could play a role in propagating neuronal dysfunction and pathology within the CNS. Nevertheless, contextualizing the role of the immune system will be difficult given contributions from genetic background, neuronal dysfunction, environmental exposure, and immunologic memory.

1.2c) Evidence for Inflammation and Role for Innate Immunity in PD

The interplay between inflammation and neuronal dysfunction is complex. Neuronal death can induce inflammation by releasing apoptotic and necrotic cellular factors that are recognized by innate immune cells. The activation of innate immune cells by these general factors could then induce an adaptive immune response that would lead to specific and selective patterns of neuronal injury. Conversely, inflammation can induce neuronal death through the production of neurotoxic cytokines or direct neuronal injury. Many mechanisms of neuronal dysfunction have been implicated in PD pathogenesis, including proteasome-mediated protein degradation, mitochondrial dysfunction, and oxidative stress [73]. Inflammation-mediated neuronal dysfunction has also been well established. Specifically in PD, midbrain DA neurons are very sensitive to cytokines such as TNFα and IFNγ [74-77]. Microglia, CNS-resident innate immune cells, densely populate the SN, and therefore could contribute to the enhanced sensitivity of this region to

inflammatory stimuli [78]. The bacterial endotoxin, lipopolysaccharide (LPS), directly activates TLR4 receptors on microglia (and other innate immune cells) to initiate an inflammatory response. Within a week after direct LPS injection into the SN of mice, significant decreases in dopamine and dopamine metabolite levels (>60%) in the striatum and in tyrosine hydroxylase immunostain-positive (TH+) neuron number in the SN are detectable. These changes are detectable even a year after the injection [79,80]. Chronic infusion of a low dose of LPS into the rat SN leads to peak microglial activation in two weeks followed by delayed and selective loss of DA neurons. Studies *in vitro* with neuron-enriched versus mixed neuron/glia cultures suggested this was due to microglial-specific activation because dopaminergic neurons do not express TLR4 [81].

Abundant evidence in humans also demonstrates a role for chronic inflammation and innate immune activation in PD. Increased levels of cytokines (including IL-1β, TGF-β, IFNγ, and IL-6) as measured post-mortem were found in the CSF and nigrostriatal regions of individuals with PD relative to age-matched healthy controls [76,82-84]. Furthermore, proteins of the complement system, a serum-mediated mechanism designed to clear antibody and various immune targets, are found in extraneuronal Lewy bodies postmortem. This finding suggests that innate immune activation occurred in association with or in response to Lewy body formation [85]. Serum levels of TNFα are elevated in PD patients and the serum levels of IL-6 correlate with Hoehn and Yahr staging [86]. Taken together, this evidence indicates that an active inflammatory process with definite innate immune involvement is ongoing in the CNS of PD patients.

Until we can identify people with PD earlier in the course of the disease, we will be unable to definitely establish whether innate immune activation is an etiologic or resultant process in the pathophysiology of disease. However, inflammation and innate immunity are also known to play an important role in various animal models of PD. In these models, it is often present before detectable neuronal dysfunction or death occurs. In the MPTP mouse model, activation of microglia, increased endothelial expression of

adhesion molecules, and infiltration of T lymphocytes can be dampened by dexamethasone treatment. Dexamethasone is a potent corticosteroid that globally suppresses immune responses. The anti-inflammatory effects of dexamethasone in the MPTP mouse model protect against DA neuron loss [87,88]. The MPTP model does not display significant blood brain barrier (BBB) disruption and thus, may not completely reflect the disease process in that respect [87]. People with PD have significant BBB dysfunction, which is discussed below. The use of anti-inflammatory drugs in neurotoxin animal models such as MPTP and 6-hydroxydopamine (6-OHDA) also attenuates DA neuron loss [89]. The direct LPS intranigral injection model is useful in understanding how direct engagement of receptors on innate immune cells can initiate a neurotoxic inflammatory response. On the other hand, direct toxin models may better reflect how neuronal death induces inflammation, which then propagates further neuronal injury [79]. Viral overexpression of human α-synuclein in the mouse SN induces inflammation and microglial activation that is followed months later by progressive death of TH+ neurons [90]. Innate immune activation is also observed in various transgenic mouse models of α-synuclein overexpression. In a mouse model, expression of wild-type α-synuclein under the direction of the Thy1 promoter resulted in microglial activation and TNFα expression in the striatum at 1 month of age and progression to the SN at 5-6 months of age [91]. These changes can persist up to 14 months of age. Other various models of transgenic mutant and wild type α-synuclein overexpression also display chronic microgliosis [92-95]. In summary, direct neurotoxin models (MPTP, 6-OHDA) typically involve necrosis of DA neurons that initiate an inflammatory response through innate immune activation, whereas in LPS and α-synuclein over-expression models, an initial inflammatory reaction precipitates neuronal dysfunction and death. It is unclear which of these two pathogenic processes predominates in PD since we are only able to detect human disease at late stages. Investigation of inflammation in the context of multiple animal models remains

extremely important because they allow investigators to clarify the interactions between neuronal dysfunction, innate immune activation, and inflammation.

Inflammation can also alter the permeability of the BBB allowing increased flux of inflammatory factors and immune cells from the periphery. Innate immune activation leads to release of proinflammatory factors that mediate this increased BBB permeability. Adaptive immune cells normally survey the CNS. Increased BBB permeability could allow for increased influx of peripheral adaptive immune cells which could then be activated by inflamed microglia as they are the primary innate immune cell of the CNS. These adaptive immune cells are activated in response to specific antigens and would be able to promote inflammation in other CNS regions that contain these same antigens. This inflammatory response could induce neuronal injury (if not already present) that in turn could propagate a positive-feedback cycle of neuronal injury and inflammation in the development of PD [77]. Thus, increased BBB permeability could be a catalytic factor in the development of PD.

In vivo evidence for increased BBB permeability has been revealed through PET measurement of ligand uptake by the molecular efflux pump, P-glycoprotein, and increased albumin in the CSF of PD patients [96,97]. Possible mechanisms for this BBB leakiness include cerebral capillary basement membrane thickening and collagen accumulation [98]. Increased proliferation of blood vessels or endothelial cells also occurs in PD [99]. Upregulation of ICAM-1, an important adhesion molecule for immune cell diapedesis, has been reported on blood vessels near the SN in postmortem brain tissue from PD patients. Endothelial upregulation of ICAM-1 is also present in MPTP-induced rodent and non-human primate PD models [100,101]. Inflammatory responses that promote BBB leakiness may promote an efflux of antigen from the CNS to lymphoid tissues where adaptive immunity can be engaged. An activated endothelium and BBB could also permit greater influx of peripheral immune cells that further propagate inflammation. Understanding the process of how innate immune cells present neuronal antigens to

adaptive immune cells and then how these adaptive immune cells are recruited to the CNS in PD will be important for developing a coherent model of disease pathogenesis. Understanding the role of inflammation in this disease will aid development of therapeutic approaches to promote neuroprotective immune responses.

Overall, it is clear that DA neurons are especially susceptible to damage from inflammatory stimuli. Furthermore, inflammation is a key pathogenic process in models of PD that often precedes detectable neuronal injury and degeneration. It will be difficult in humans to determine whether inflammation precedes neuronal injury until earlier diagnosis or biomarkers are established. Nevertheless, the brains of PD patients display chronic neuroinflammation that could be targeted to modify disease progression. The selective loss of certain neuronal populations while others are spared could point to a role for adaptive immunity in mediating this inflammatory process.

1.2d) Engagement of Adaptive Immunity: Microglial Activation and MHC in PD

Some of the earliest evidence for inflammation in PD is from identification of chronic microglial activation in post-mortem brains. Microglia are CNS-resident immune cells that play an important role in neurologic development and maintenance. They are critical for recruitment of peripheral immune cells through secretion of chemokines and thus, amplify inflammatory signals in the CNS. These inflammatory signals could originate from neuronal dysfunction or external insults (trauma, toxicants, etc.). Microglia respond to numerous signals such as bacterial and viral products, α-synuclein, complement, antibodies, and cytokines [102-105]. Neuronal death may trigger microglial activation through loss of inhibitory CD200:CD200R signaling, ligation of microglial receptors that recognize factors released during apoptosis, release of α-synuclein, and/or through binding of complement or antibodies bound to neurons [103,104]. In response to these stimuli, microglia produce cytokines [104,106,107], reactive oxygen species [108,109], prostanoids that have immunomodulatory functions [110], and chemokines that recruit

peripheral immune cells [111,112]. Neuronal death can occur in response to cytokines such as TNFα, to ligation of death receptors such as Fas, and to toxicity of reactive oxygen species, as well as phagocytosis [104,111]. Like other cells with antigen-presentation functions (B lymphocytes, macrophages, monocytes, dendritic cells), activated microglia will express Major Histocompability Class II (MHC-II) molecules that present endocytosed or lysosomal peptides to CD4 T lymphocytes (**Fig 2**). MHC-II molecules are α/β heterodimers that require binding of a peptide approximately eight to ten amino acids in length to be stably expressed on the surface of activated antigen-presenting cells. In humans, MHC-II molecules include HLA-DR, HLA-DQ, and HLA-DP. The majority of these peptides are derived from the processing of endocytosed proteins or from the typical protein constituents of endolysosomes. MHC-II genes are commonly known for their use in tissue typing for organ donation. Recognition of a specific peptide:MHC-II complex by a cognate CD4+ T cell through its unique receptor will activate the T cell to allow it to perform its effector functions: proliferation, cytokine secretion, assisting antibody-secreting B cells to produce higher affinity antibodies through a process called hyperaffinity maturation, and allow for proper activation and maintenance of cytotoxic CD8 T cells [113-116]. Presentation of antigens on MHC on the surface of activated microglia or other infiltrating innate immune cells is the key to engagement of adaptive immunity, which in turn can propagate the inflammatory process. MHC expression has been well-documented in PD.

Microglial expression of MHC-II molecules has been reported in states of chronic neuroinflammation, including neurodegenerative diseases. In the healthy CNS, microglial MHC-II expression is difficult to detect. In PD brains, HLA-DR immunostain-positive microglia are found throughout the nigrostriatal tract and other parts of the CNS, including the hippocampus, entorhinal cortex, and cingulated cortex [117-119]. In those individuals exposed to MPTP through the use of contaminated heroin, microgliosis is present even years after exposure [120]. Chronic microglial activation is also seen years

after initial MPTP administration in primate models of PD irrespective of acute or chronic dosing [121-123]. Positive correlations between disease duration and the microglial/macrophage activation marker CD68 and between MHC-II expression and amount of α-synuclein deposition in the SN of post-mortem human brain sections have been reported [102]. These activated microglia are associated with damaged neurons and Lewy body pathology [119]. *In vivo* PET imaging of PD patients demonstrates that microglial activation is concentrated in the SN and also occurs throughout the diseased CNS. Microglial activation does not seem to progress over the course of two years, suggesting that it is sustained at a constant level during clinically detectable disease and likely plays a role early in disease [124]. PET studies have also correlated microglial activation in the human midbrain with increased motor score on the Unified Parkinson's Disease Rating Scale (UPDRS) [125]. Microglial activation is prevalent in PD and occurs early in the disease process, suggesting that inflammatory processes and microglial effector functions play a prominent role in promoting disease development. Microglial effector functions could engage adaptive immunity in the context of antigen presentation on MHC to amplify and propagate inflammation.

1.2e) Adaptive Immunity (I): T Lymphocytes in PD

Given evidence for homeostatic surveillance of the CNS by naïve and memory T cells, these lymphocytes could be involved in both initiating and propagating steps of PD pathogenesis [126,127]. In both PD patients and animal models, infiltration of T cells into the SN has been demonstrated [100,117,118]. In conjunction with HLA-DR expression on microglia, T cell infiltration has been shown in post-mortem brain sections from PD patients. This evidence suggests potential direct interaction with antigen-presenting functions of microglia [117,118]. Furthermore, levels of β2 microglobulin, a protein required for stability of MHC-I molecules, are increased in the striatum of PD patients [83]. MHC-I molecules activate CD8 T cells by presenting cytosolic peptides processed through the

proteasome pathway (**Fig 2**). Unlike MHC-II, MHC-I molecules are ubiquitously expressed on all cells (including neurons) except erythrocytes. Upon engagement of peptide: MHCI, CD8 T cells can directly kill cells by engagement of death receptors via Fas or TNFα or by direct lysis through release of granzymes and perforin. In addition to CD4 T cell-mediated toxicity, direct neuronal injury may be caused by CD8 T cells that are reactive against neuronal antigens presented on MHCI molecules [128]. Thus, T-cell mediated neurotoxcity may be driven by direct cell lysis, engagement of cell death receptors, and cytokine secretion through recognition of peptide:MHC molecules on innate immune cells.

To better understand if adaptive immune responses are altered in PD patients, a handful of studies have examined the composition of T-cell subsets in the peripheral blood. PD patients are reported to have decreased overall numbers of lymphocytes without a change in frequency [129,130]. Compared to people with other neurologic diseases, individuals with PD have increased memory T cells but decreased naïve T cells [131]. Memory T cells respond faster and with greater magnitude than activated naïve T cells. A more activated, cytotoxic T cell response is suggested by decreased CD4:CD8 ratios and a shift to more IFNγ– versus IL-4-producing T cells in PD patients [129,130,132]. Within the CD4 T cell compartment, PD patients have an increase in CD45RO+ T cells, which represent an activated and/or memory T cell population [133]. CD45RO+ expression also positively correlates with UPDRS motor score. Naïve CD4 T cells have increased Fas expression, which may explain why their frequency is decreased [130]. Fas expression is normally upregulated after T-cell activation and ligation by FasL induces apoptosis. This process is used on T lymphocytes to maintain immune privilege or initiate contraction after an immune response [134]. Increased memory and activated CD4 T cells in conjunction with a relative increase in CD8 T cells could suggest an active inflammatory process. Conflicting reports of changes in relative frequencies of CD4+CD25+ T cells in peripheral blood exist but without further characterization of this subpopulation, the

biological significance of this is unclear [129,130,132,135]. CD25 is increased on activated or memory T cells, as well as regulatory T (T_{reg}) cells. One study that demonstrated that CD4+CD25+CD127- regulatory T cells from PD patients had less suppressive capacity compared to cells from healthy controls [133]. The relative number of effector to regulatory T cell responses is thought to regulate immune responses. Decreased effectiveness of regulatory T cells could promote a chronic neuroinflammatory state or allow for breaking of tolerance to neuronal antigens that would lead to abnormal immune responses against CNS proteins. Investigation of why these regulatory T cells have reduced suppressive capacity could also provide insight into PD pathogenesis and disease progression.

Despite information about changes in relative frequency of T cell subsets, little is known about the identity of CNS antigens to which activated and memory T cells in people with PD are responding. One study looked at whether specific T cell receptor genes are preferentially expressed in PD patients and reported that CD8 T cells have a lower frequency of Vβ8 expressing cells [136]. The use of deep sequencing techniques to identify patterns of T cell receptor usage in PD patients could provide evidence for response to specific antigens and help to identify those antigens. Identification of such patterns could also be a useful biomarker. T cell biomarkers have been investigated and some candidate proteins have been identified within these cells such as β-fibrinogen and transaldolase [137]. The pathogenic relevance of these changes is not yet understood.

Other overall pathogenic changes in peripheral blood lymphocytes from PD patients have been reported. Lymphocytes from PD patients display an increased incidence of micronuclei, single strand DNA breaks, and oxidized purine bases [138]. Interestingly, levodopa treatment seems to reduce this DNA damage in peripheral blood lymphocytes [139]. Markers of apoptosis, caspase-3, and Cu/Zn superoxide dismutase activity are increased in lymphocytes from people with PD [140]. This DNA damage and increased level of apoptosis could be representative of a systemic, pathogenic process involving oxidative stress, specific immune responses, and/or intrinsic, genetic factors.

The use of animal models to assess the contribution of lymphocytes will inform us of how these *in vivo* human changes may contribute to disease etiology and progression.

The contribution of T cells to PD-like pathology in animal models has been assessed but mainly in direct toxin models. CD8 T cells are seen in greater amounts than CD4 T cells in the acute MPTP neurotoxin model. In this model, these lymphocytes have increased expression of LFA-1, an integrin that binds endothelial adhesion molecules, to allow for diapedesis across the BBB [87]. DA neuron loss and behavioral deficits caused by both chronic and acute MPTP administration are attenuated in *RAG2-/-* mice that lack both T and B cells [100,141]. Mice in which there is a global loss of αβ-T cells through knockout of the T cell receptor β-chain, as well as mice lacking CD4 T cells (*CD4*-/-) are also protected in the MPTP model [100]. The loss of CD8 T cells was not protective. Reconstitution of *Rag1-/-* mice with FasL-mutant splenocytes attenuated DA neuron cell death while reconstitution with *IFN*γ*-/-* splenocytes did not. These findings suggest that Fas-FasL interactions involving CD4 T cells play an important role in promoting MPTP-induced neurodegeneration. As discussed above, cytotoxic T cells use FasL to induce apoptosis in target cells but Fas receptor is also important in T cell homeostasis and contraction. Impaired FasL-Fas interactions result in unchecked T cell activation and proliferation. An extreme example manifests itself in autoimmune lymphoproliferative syndrome caused by unchecked T cell proliferation [142]. Dysregulation of this pathway may also leave chronically activated macrophages or microglia unchecked allowing for propagation of the inflammatory response. Fas-FasL signaling may also indirectly be contributing to neuronal damage by inducing Fas-induced apoptosis-resistant astrocytes to produce proinflammatory cytokines that damage neurons [143]. In the intranigral AAV-human-α-synuclein overexpression mouse model, B and T lymphocyte infiltration in the SN persists after peak of microglial activation suggesting that adaptive immune cells propagate inflammation in this model as well [90]. One study with MPTP gives a potential model of adaptive immune engagement where nitrated α-synuclein drains from the CNS

into cervical lymph nodes. Robust T cell responses were initiated when mice were immunized with the C-terminus of the nitrated α-synuclein. Transfer of T cells from immunized mice into MPTP-treated mice enhanced neuroinflammation to a slight but significant degree [144]. T cells from mice immunized with nitrated α-synuclein produced mostly IL-17 and TNFα after *ex vivo* restimulation. Adoptive transfer of Th17-polarized T cells from nitrated α-synuclein-immunized mice into wild-type mice greatly exacerbated MPTP-induced neurodegeneration. Neurodegeneration was not exacerbated by adoptive transfer of T cells polarized to Th1 (IFNγ-producing) or Th2 (IL-4-producing) phenotypes. Adoptive transfer of *ex vivo* differentiated regulatory T cells into MPTP-induced mice attenuated neurodegeneration [112]. The novelty of this study is that it demonstrates that modulation of the adaptive immune response can indeed determine the outcome of neurodegeneration in a model of PD.

Given this evidence, T cell responses to modified α-synuclein or other modified antigens from DA neurons could initiate an adaptive immune response that propagates neuronal death. These responses would differ based on the context of antigen presentation by innate immune cells. Various etiologies could allow for the adaptive immune system to be exposed to modified α-synuclein (i.e. toxins) or increased escape of CNS antigen (i.e. head trauma). These mechanisms would break normal tolerance mechanisms by presenting self-antigens that are cross-reactive with cognate peptide:MHC of existing memory T cells. These antigens could also activate naive T cells by presenting self-antigens for which T cells are not normally negatively selected against or tolerized to in the context of an inflammatory response. Further studies to identify specific antigens that T cells respond in animal models and humans will be critical to understanding the role of the adaptive immune system in pathogenesis of PD.

1.2f) Adaptive Immunity (II): Antibodies and B Lymphocytes in PD

B lymphocytes are the antibody-secreting cells of the immune system. They are the key mediators of humoral immunity in that their secreted immunoglobulins can have effects at sites far from the actual site of secretion [70]. Unique antibodies, as well as increased antibody levels against CNS proteins are present in people with PD [145,146]. However, infiltration of antibody-producing B lymphocytes into the CNS has not been not reported in brains of PD patients [100,117,118]. While infiltration of B lymphocytes is present under some inflammatory conditions, the aggregation of antibodies at a specific site is enough to promote inflammation. Antibodies can precipitate inflammatory reactions through activation of the complement system or effector cells through surface immunoglobulin receptors. B cells can be activated to secrete antibody upon engagement of their surface immunoglobulin receptor in the context of inflammatory cytokines from innate immune cells or direct recognition of stimuli through pattern recognition receptors. These lymphocytes also can re-engineer the antibodies they secrete through a process called affinity maturation that occurs in secondary lymphoid organs such as the spleen or lymph nodes. Particularly for PD, there is some evidence that the meninges can act as a secondary lymphoid organ [147,148]. Affinity maturation is mediated by activated T cells and occurs through interactions between the T cell receptor and peptide-MHC-II on the surface of the B cell. B cells that have undergone affinity maturation produce antibodies of higher affinity to their target and can become memory cells that are long-lived [70]. B cells in this manner have a unique niche in the adaptive immune system that depends heavily on signals from innate immune cells and CD4 T cells.

A decrease in the number of peripheral blood B cells in PD patients has been reported but the significance of this finding needs to be contextualized [129,130]. Reduced peripheral blood B cell counts in other autoimmune or inflammatory diseases are due to decreased circulating memory B cells. This reduction in B cell number may be related to active inflammation and cellular activation [149-151]. Increased study is needed to

understand the role for B cells in PD outside of their antibody-secreting functions discussed below.

Antibodies against DA neurons have been identified in PD patients that are not present to the same extent in healthy people [145,146,152,153]. At present, the lack of a universal antibody or "PD"-specific antigen refutes an etiologic role for B cells and antibodies but does not rule out either a pathogenic or a protective role for B cells in modulating inflammation in PD. It is also possible that a universal antibody or antigen in PD does not exist because no single antigen is required for disease induction. On the other hand, we may not able to identify one because we only assess antibody production at a late stage of disease. Within human PD brain sections, immunoglobulins are found to colocalize with pigmented DA neurons in close proximity to FcγR+ microglia. The number of IgG+ neurons positively correlated with the number of MHCII+ microglia suggesting a link between antibody-mediated destruction of neurons and antigen presentation. Further supporting this link, these microglia contain pigmented granules suggesting antibody-mediated phagocytosis of pigmented DA neurons [154]. A pathogenic role for anti-neuronal antibodies in PD is further supported by a study using immunoglobulins from PD patients in a rat model. These immunoglobulins were injected into the SN of rats resulting in a loss of TH+ neurons while acetylcholinesterase-positive neurons in the medial septal region were spared [155]. These immunoglobulins induced perivascular inflammation, microgliosis, and loss of TH+ neurons in the SN that was dependent on expression of the Fcγ receptor [156]. Similarly, Fcγ receptor-/- mice are resistant to chronic but not acute MPTP administration [141]. One caveat to these results is that the deletion of Fcγ receptors in mouse models impacts numerous cell types (monocytes, microglia, neutrophils, basophils, mast cells, NK cells) and reduces the ability to respond to cellular bound IgG through multiple mechanisms. These mice are also resistant to neurodegeneration and microglial activation induced by AAV-induced intranigral expression of human wild type α-synuclein [157]. The AAV- α-synuclein model

also leads to significant IgG deposition, suggesting that humoral immunity and/or breakdown of the BBB is playing a role [90]. These findings suggest that with DA neuron injury, the humoral arm of the immune system can play a role in chronic neuroinflammation.

Given the chronic inflammatory nature of PD, humoral immunity could play an important role in the progression of PD while T cell immunity may be more important for disease onset. Intuitively, this idea makes sense because CD4 T cell activation is essential for potent engagement of humoral immunity. In contrast, antibodies may also play a protective role. The use of a mouse antibody against α-synuclein showed that microglia can take up antibody-bound α-synuclein *in vivo* and prevent neuron to astrocyte transmission of α-synuclein [158]. It is unknown whether this phenomenon can happen in the PD brain but it raises the distinct possibility that certain sets of antibodies could play a protective role. Pathogenic antibodies could promote inflammatory reactions or propagate α-synuclein spread through uptake by Fc receptor-expressing cells which would then seed of a-synuclein and propagate inflammation. Other antibodies may be protective by aiding in clearance of α-synuclein aggregates.

There have been a few attempts to use antibodies as biomarkers for PD. One study found decreased levels of anti-α-synuclein antibodies in PD patients but not in controls or patients with other neurodegenerative conditions with sensitivity and specificity of 85 and 25%, respectively. These antibodies did not correlate with age, duration of disease, or Hoehn and Yahr staging [145]. The presence of α-synuclein antibodies may hint at pathologic mechanisms but are not specific enough to be used diagnostically. Another study demonstrates the ability to use 10 autoantibody biomarkers to differentiate PD from normal aging, Alzheimer's disease, breast cancer, and multiple sclerosis with accuracies over 85% [146]. Another report describes that in people with sporadic PD, the frequency of those with anti-α-synuclein antibodies is not significantly different from healthy controls. However, around 90% of people with familial PD have

these antibodies. The authors suggest that the antibodies in familial PD may represent more pathogenic epitopes rather than incidental ones [153]. Studies using antibodies from patients at earlier stages of disease will likely give more insight into the role of antibodies in PD pathogenesis. Such antibodies are less likely to be incidental from the release of CNS antigens as inflammation propagates in disease.

1.2g) Antigen Presentation as an Etiologic Factor for PD

Further implicating inflammation in PD pathogenesis, single nucleotide polymorphisms (SNPs) in the MHC-II locus have been associated with increased risk of developing PD. In particular, people homozygous for a risk-conferring SNP, *rs3129882,* in the first intron of *HLA-DRA* have a 1.7 fold higher risk for PD than individuals homozygous for the non-risk conferring allele [159,160]. Other SNPs in the MHC-II locus associated with PD have also been reported in the *HLA-DRB1* and *-DRB5* genes. Another study has shown an association with the HLA-DQB1*06 allele in a German cohort [161-165]. The up-regulation of MHC-II mRNA (*HLA-DRA, HLA-DPA1, HLA-DQA1*) and increased expression of HLA-DR on monocytes in cerebrospinal fluid (CSF) has been reported [131,166]. Further study is needed to assess whether changes in MHC expression occurs in PD and in particular in association with the *rs3129882* SNP. These associations between PD and the MHC-II locus suggest that certain antigens or patterns of MHC-II expression could promote the development of PD. The quality of the interaction between CD4 T cell receptors and peptide:MHC-II impacts CD4 T cell expansion and differentiation [114,116] (**Fig 3**). Thus, MHC-II expression could modulate the overall quality of the inflammatory response via activation of the adaptive immune system. Individuals with a certain MHC-II expression pattern or haplotype could propagate inflammation initiated by neuronal dysfunction or death more readily. Activation of the adaptive immune system by innate immune cells could allow for propagation of chronic inflammation in response to particular stimuli and antigens. Such a mechanism would promote or exacerbate PD

progression. The evidence for involvement of T cells, B cells, and antibodies in promoting inflammation in PD is discussed below.

Certain antigens or environmental exposures could synergize with a person's MHCII genome to put them at risk for developing PD. In this manner, the immune system would be an etiologic factor. Peptides presented on MHCII would activate T cells to influence neuronal function through cytokine secretion, which may promote cytotoxicity. This idea is supported by the association between PD and autoimmune diseases, such as bullous pemphigoid, systemic lupus erythematosus, and Sjogren's syndrome [61,62,65,67]. Gut inflammation is hypothesized to play a role in the development of sporadic PD. Overrepresentation of *CARD15* mutations associated with Crohn's disease and *Helicobacter pylori* seropositivity before age 75 in people with sporadic PD supports this hypothesis [167,168]. A GWAS identified polymorphism in the *LRRK2* gene is associated with Crohn's disease. Mutations in this gene give rise to an autosomal dominant form of PD (see below). Given the extensive neuronal network of the GI tract, its constant interaction with the environment, and its extensive, unique immunological repertoire, the GI tract represents a potentially interesting site for initiation of PD pathogenesis because of the complex, continuous interplay between the innate and adaptive immune system through antigen presentation.

Immune responses to infections are also implicated in PD through association with influenza, toxoplasmosis, and Epstein-Barr Virus (EBV) [66,68,169]. Post-encephalitic parkinsonism after the 1918 Spanish Flu pandemic and a cross-reactive antibody between EBV and α-synuclein suggest that adaptive immune responses to certain viruses may predispose an individual to PD [66,169]. Infection of mice with the A/VN/1203/04 strain of the H5N1 influenza virus demonstrated viral replication in the CNS, microglial activation and increased proinflammatory cytokine and chemokine expression lasting 90 days after viral clearance. A transient decrease in tyrosine hydroxylase (TH) expression in the SN was also observed [170]. This virus can also induce α-synuclein phosphorylation and

aggregation [171]. Japanese Encephalitis Virus (JEV) has also induced a post-encephalitic parkinsonism with similar neuropathologic features and locomotor dysfunction as that seen in people with sporadic PD [172]. JEV infection in rats results in hypokinesia attributable to depletion of catecholamines in the CNS [173]. These studies provide provocative evidence that an adaptive immune response could induce PD-related neurologic dysfunction initiated solely by inflammation. MHC expression on stimulated innate immune cells would play a critical role in determining which antigens are presented to the adaptive immune system. Activated adaptive immune cells would then propagate the inflammatory reaction. The use of animal models of PD such as the human α-synuclein-expressing adenoassociated virus (AAV-Syn) model [90] that more directly engages adaptive immunity could help address the validity of these associations. Global loss of MHC-II in a mouse model is protective against neurodegeneration, microglial activation, and T cell infiltration when human α-synuclein is virally overexpressed in the SN. While this model lacks CD4 T cells and has impaired antibody production, it does provide interesting evidence for a role of CD4 T cells and MHC-II expression in PD pathogenesis [174]. Other neurotoxin models employing MPTP or 6-hydroxydopamine (6-OHDA) may be less likely to engage adaptive immune mechanisms because they are mainly driven by neuronal toxicity rather than a more direct inflammatory stimulus. Clarifying whether the immune system acts as an etiologic factor in PD or simply propagates inflammation initiated by neuronal dysfunction will be an important part of determining PD etiology and pathogenesis.

1.3) Figures

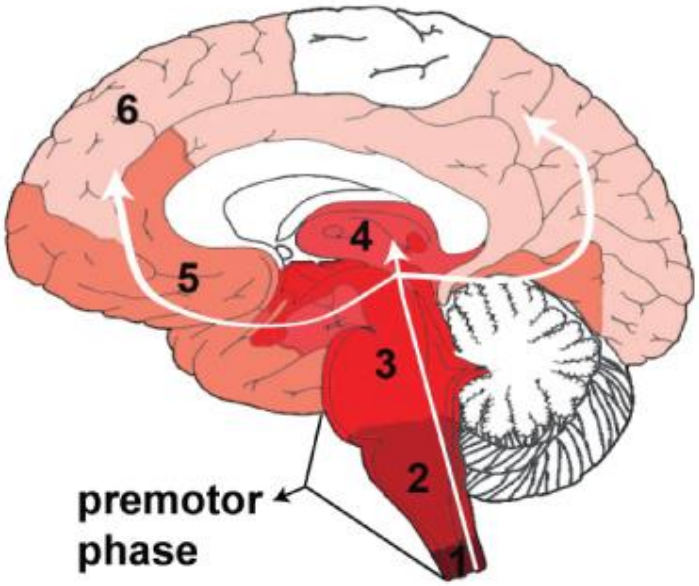

Figure 1. The Braak Hypothesis proposes a pattern for progression of disease pathology. Pathology initiates in the periphery and progresses into the CNS via the vagus nerve and/or olfactory nerve in a predictable, progressive manner. Numbers indicate proposed sequence of progression into various brain regions.

Genome Wide Association-Based Loci			
Chromosome	RefSNP #	Gene	Odds Ratio
1q22	N370S	GBA	3.08
4p16	rs11248060	DGKQ	1.69
4q22	rs356229	SNCA	1.35
6p21	rs3129882	HLA-DRA	1.31
21q21	rs2823357	USP25	1.15
12q12	rs1491942	LRRK2	1.19
17q21	rs1724425	MAPT	0.68
4p15	rs4538475	BST1	1.25
3q27	rs11711441	LAMP3	0.82

Adapted from Ross OA, *Curr Genet Med Rep*, (2013), 1:52-57.

Table 1. Genetic polymorphisms associated with sporadic forms of PD

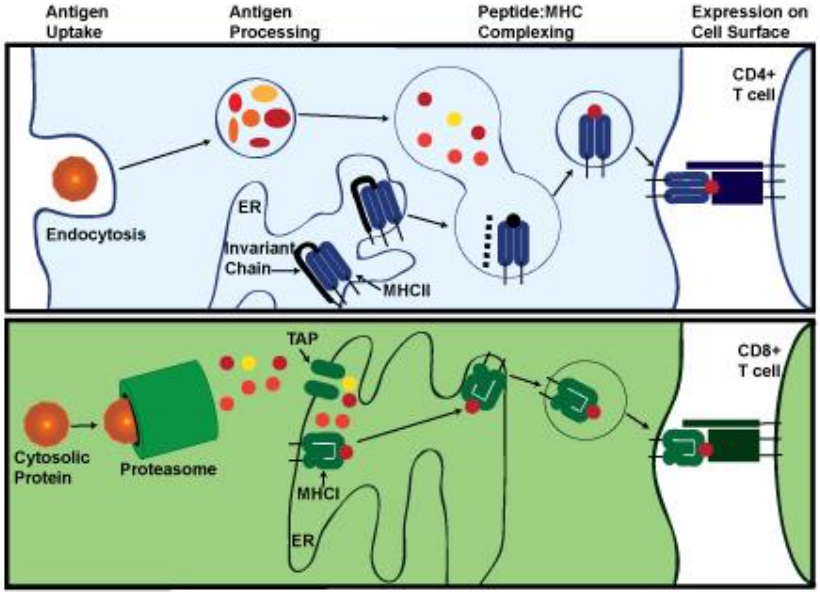

Fig 2. Biosynthesis of peptide-MHC complexes. MHC-II peptides are derived from endocytosed antigens while MHC-I proteins are derived from proteasome-processed cytosolic proteins. MHC-II presents peptides to CD4 T cells while MHC-I presents peptides to CD8 T cells.

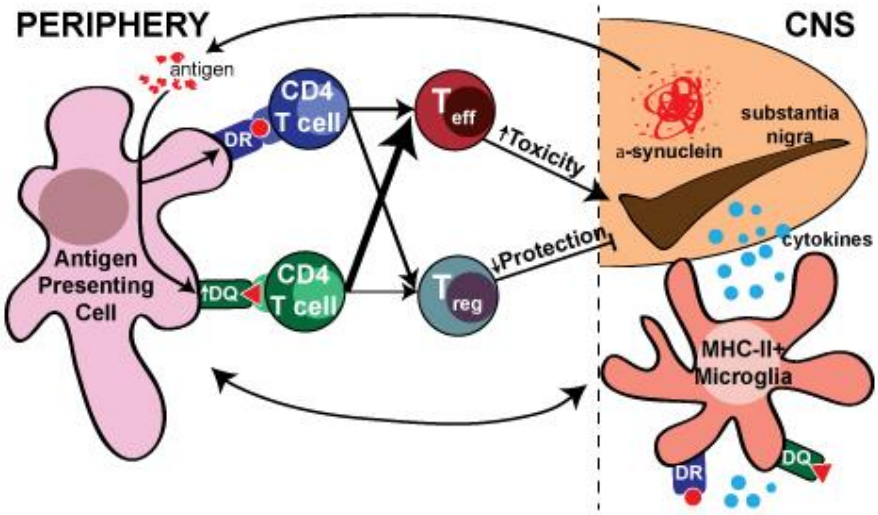

Fig 3. Model of MHCII mediated antigen presentation to CD4 T cells. Antigen (potentially α-synuclein) from the CNS is presented by either HLA-DR or –DQ

molecules in secondary lymphoid tissues to activate and differentiate CD4 T cells. Failure to induce the appropriate regulatory (Treg) or effector (Teff) T cells could contribute to dopaminergic neurondegeneration in PD.

Chapter 2: Age-related changes in Regulator of G-protein Signaling (RGS)-10 expression in peripheral and central immune cells may influence risk for age-related degeneration

Previously published work[175].

2.1) Introduction

Aging is the largest risk factor for neurodegenerative diseases such as age-related maculopathy and Parkinson's disease [176,177]. The convergence of multiple mechanisms accounts for this risk: protein aggregation, mitochondrial dysfunction, oxidative stress, and inflammation [77,178]. The role of the Regulator of G Protein Signaling (RGS) 10 in modulating interactions between the immune and nervous systems makes it an interesting target to study in the context of aging [179]. The locus encoding the RGS10 protein on chromosome 10q26 has been associated with age-related maculopathy, a disease of retinal degeneration with significant microgliosis [42,180]. A polymorphism in *RGS10* has also been associated with schizophrenia [43]. Given these associations in humans, it is particularly interesting that the global loss of RGS10 in the mouse leads to microgliosis and susceptibility to degeneration of DA neurons in the midbrain in response to peripheral low dose administration of lipopolysaccharide [46,47]. This unique phenotype implicates RGS10 as a potential regulator of neuroimmune interactions and raises the question of its role in aging.

RGS10, the smallest of the RGS proteins, belongs to the R12 subfamily and is highly expressed in the brain, thymus, and lymph nodes [3,25,31,181]. The physiologic substrates of RGS10 have not been identified, but in heterologous assays it is known to selectively accelerate the GTPase activity of $G\alpha_{i3,}$ $G\alpha_{q,}$ $G\alpha_{z}$ [25]. Aging has been shown to affect the repertoire and function of G-protein coupled receptors (GPCRs) and proteins [23]. GPCRs are involved in controlling critical cellular and physiological functions. GPCRs signal

through heterotrimeric G-proteins that consist of α subunit and βγ heterodimer [182,183]. RGS proteins contain an evolutionarily conserved RGS domain that interacts with $G\alpha_i$, $G\alpha_{q/11}$, $G\alpha_{12/13}$ or Gαs subunits with variable selectivity, to accelerate intrinsic GTPase activating function of the Gα subunits therein [181,184,185]. Age-associated changes in GPCRs and G-proteins vary throughout the body but the expression of most GPCRs and G-proteins decrease with age in the brain [183,186-188]. In human lymphocytes and neutrophils, there were a variety of changes in the pattern and the quantity of G proteins with aging [189]. It has been also reported that Gα subunits may undergo age-related changes that impair coupling to G-proteins after agonist-binding. This impaired coupling would decrease the proportion of high affinity receptors that could be formed [23]. In turn, age-related changes in G-protein signaling could help explain dyregulation in numerous physiologic and cellular systems that occur with aging.

In addition to human disease associations and the sensitivity of nigral DA neurons to LPS-induced degeneration, previous work from our group and others have implicated RGS10 in immune cell and neuronal function. Specifically, we reported that RGS10 negatively regulates Nuclear Factor-κ-light-chain-enhancer of activated B cells (NF-κB) signaling, explaining the pro-inflammatory phenotype of RGS10-/- microglia [47]. In dopaminergic (DA) neurons, RGS10 plays a neuroprotective role through interactions with the Protein Kinase A (PKA)/cAMP response element-binding protein (CREB) pathway [29]. We also demonstrated that loss of RGS10 induces a dysregulated phenotype in peripheral macrophages [30]. RGS10 has also been implicated as a negative regulator of chemokine-dependent adhesion via the Vav1-Rac1-dependent pathway [28]. In platelets, RGS10 binding to Src homology 2 domain-containing phosphatase 1 (SHP-1) negatively

regulates platelet activation through a sphinophilin-dependent pathway [190]. The SHP-1 pathway is also very important in immune cells as a negative regulator of activation. The role of RGS10 in modulating these pathways could provide an explanation for the neurodegeneration seen in RGS10-/- mice after chronic peripheral LPS administration. Given that age is the strongest risk factor for neurodegeneration and that RGS10 plays a role in modulating many pathways involved in neuroimmune interactions, we hypothesized that RGS10 would play an important role in altering the regulation of DA neurons and immune cell populations in aging.

2.2) Materials and methods

2.2a) Animals

Generation of RGS10-/- mice (C57/B6) has been described previously [47]. Three to seven month old (young) mice and 18-22 month old (aged) mice were used for experiments. Age- and gender- matched wild-type (WT) mice were used as controls. Experimental procedures involving use of animal tissue were performed in accordance with the NIH Guidelines for Animal Care and Use and approved by the Institutional Animal Care and Use Committee at Emory University School of Medicine in Atlanta, GA. Unless noted, mice were euthanized by intraperitoneal Euthasol injection.

2.2b) Flow Cytometry

From mice, spleens were homogenized into a single-cell suspension and blood was collected in EDTA-coated tubes by cheek bleed and then red blood cells (RBCs) were lysed with RBC lysis buffer (1.5M NH_4Cl, 0.1 M $KHCO_3$, Na_2EDTA, pH 7.4) For

surface staining, cells were washed with FACS buffer and then stained for 20 minutes with fluorophore-conjugated antibodies. For mouse tissues, the antibodies used wereanti-CD11b-PE (ebiosciences), anti-Ly6G-FITC (ebiosciences), anti-CD45-APC (ebiosciences), anti-CD3ε-PE-Cy7 (ebiosciences), anti-CD8α-APC-Cy7 (ebiosciences), anti-CD4-V500 (BD Biosciences) and anti-CD16/CD32 (ebiosciences). If applicable, intracellular staining was then performed using Invitrogen Fixation and Permeabilization Media with goat anti-RGS10 primary antibody (Santa Cruz Biotechnology) and donkey anti-goat Fc FITC-conjugated secondary antibody (Santa Cruz Biotechnology). If intracellular staining was not performed, cells were washed and then fixed with 1% paraformaldehyde for 30 minutes. After washing, cells were stored in FACS buffer until analysis on a LSR-II flow cytometer (BD Biosciences). Data analysis was performed on FlowJo software.

2.2c) Immunofluorescence and Image Quantitation

Mice were anesthetized with 200 mg/kg Euthasol (Virbac Animal Health, Fort Worth, TX) and brains extracted and fixed for 24 hours in 4% paraformaldehyde. Brains were sectioned onto glass slides (Leica Cryostat CM3050 S). Sections on glass slides were fixed for an additional 15 min in 4 % paraformaldehyde, followed by a 1 X PBS rinse, pH 7.4. Sections were incubated in 0.2 M glycine, pH 7.4, for 30 min to minimize tissue autofluorescence caused by the aldehyde fixative. Sections were permeabilized for 35 min in Tris-buffered saline (TBS) containing 0.3 % Triton X-100 and 1 % normal donkey serum (NDS), followed by blocking for 60 min in TBS containing 1 % NDS. Sections were incubated in primary antibody for 24 hrs at 4 °C in TBS containing 0.1 %

Triton X-100 and 1 % NGS. Iba1 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (1:250), RGS10 (C-20, Santa Cruz) (1:200) followed by the appropriate Alexa-conjugated secondary antibodies (1:500, Invitrogen). Non-immune IgG sera at the same concentration as the primary antibodies were used to confirm the specificity of staining. To quantify the level of RGS10 on Iba1+ cells, 5-6 sections per animal from young and aged wild-type mice (n=3) were selected between bregma -1.28 mm and -2.12 mm. Images of RGS10+ or Iba1+ cells from 20 random fields of brain sections were captured under 20x objective lens on a Nikon 90i fluorescence microscope using thresholding analysis on Nikon Elements 5 software. RGS10 expression in Iba1+ cells was quantified in these images using ImageJ software. Total RGS10 expression intensity in fields was quantified by thresholding on the mean intensity value + 80 intensity units of Iba1 fluorescence intensity. The average RGS10 expression intensity and average number of RGS10-immunoreactive pixels per Iba1+ cell was quantified after thresholding on the mean intensity value + 80 intensity units of Iba1 fluorescence intensity and then using the Analyze Particles function.

2.2d) Cerebrospinal fluid (CSF) and Serum collection

Mice were anesthetized with mixtures of Ketamine (100 mg/kg), Xylazine (10 mg/kg) and Acepromazine (2 mg/kg) and placed into a stereotaxic frame with the nose pointed down. Then, a small gauge needle was inserted into the cisterna magna (the triangular space between the back of the cerebellum and the medulla oblangata). A silastic tube was attached to the needle to drain CSF into a test tube. CSF was visually checked for blood contamination and not used in analysis if fluid was opaque, cloudy, or colored. Blood

was collected into EDTA-coated tubes by the cheek bleed method using a lancet to puncture the submandibular vein. Serum was separated from blood samples using Microvettes® 200 Z-Gel (SARSTEDT) by centrifugation at 10,000 rpm for 5 min at room temperature.

2.2e) Multiplexed ELISAs

Serum and CSF were analyzed for cytokines and chemokines (mouse IFN-γ, IL-1β, IL-6, IL-10, IL-12, KC, and TNF) using a multiplexed immunoassay per the manufacturer's instructions (Meso-Scale Discovery, Gaithersburg, MD).

2.2f) Immune Cell Isolation from Adult Mouse Brain

Microglia were isolated from adult mice as described previously [191] . Briefly, brain was finely minced using a scalpel and digested using a papain-dispase solution at 37°C. Microglia and other immune cells were separated from myelin, red blood cells, and other cell debris by collecting the 30:37 layer interface of a 30:37:70 Percoll gradient. Immune cells were washed out from Percoll with 1x Hank's Balanced Salt Solution by density centrifugation.

2.2g) Western Blot Analysis

Striatum and/or ventral midbrain tissues were dissected from RGS10 WT or-/- animals. Tissues were sonicated in 2% SDS/8M Urea. Total protein extracts were then quantified using the bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL, USA) and bovine serum albumin as the standard. Equal amounts of protein extracts

were resolved by SDS–PAGE and analyzed by immunoblot using rabbit polyclonal Anti-Tyrosine Hydroxylase Antibody (AB152; Millipore), mouse monoclonal α-tubulin antibody (Calbiochem), and goat polyclonal RGS10 (C-20) antibody (Santa Cruz Biotechnology). Images were quantified using GeneTools image analysis software (Syngene).

2.2h) Quantitative Real-time RT-PCR (QPCR)

Striatum and/or ventral midbrain tissues were dissected from RGS10 wild-type WT or RGS10-/- animals and frozen on dry ice. Total RNA was extracted using TRIzol reagent (Invitrogen) and purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Up to 2 ug of each RNA sample was treated with DNase I and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. QPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and previously validated QPCR primers for murine GAPDH (forward: 5'CAAGGTCATCCATGACAACTTTG3'; reverse: 5'GGCCATCCACAGTCTTCTGG3'), Nrf2 (forward: 5'CCCGGTTGCCCACATTC3'; reverse: 5'TGTCTCTGCCAAAAGCTGCAT3'), Parkin (forward: 5'AGCCCTCCAAGGAAACCATC3'; reverse: 5'CGTTTTTTTCAATTGGCACGT3'), and TH (forward: 5'TTGGCTGACCGCACATTT3'; reverse: 5'GCCCCCAGAGATGCAAGT3') genes. Reactions were performed in triplicate using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) and data were analyzed by the ΔΔ Ct method.

2.2i) Dopamine Metabolism Measurement

Neurochemical analysis was performed as previously described [192] with slight modifications. Briefly, mice (n = 4 per age group) were sacrificed and the brain was placed in a mouse brain matrix (Asi-Instruments). Coronal sections (2 mm) of the brain were cut by placing razor blades in each channel in the matrix. The coronal sections containing the striatum were placed on a cooled plate, and the striatum was dissected out. Tissue was weighed and homogenized in 10 volumes of ice-cooled 0.1 M perchloric acid (containing 347 μM sodium metabisulfate, 134 μM EDTA,) and then centrifuged at 4 °C for 15 min at 10,000×g. The supernatants were filtered using 0.2 μm filters (Fisher) for 2 min at 10,000×g. Dopamine (DA), and its metabolites 3-4-dihydroxphenylacetic acid (DOPAC) and homovanillic acid (HVA), and 3-methoxytyramine (3-MT) were analyzed using reverse-phase ion pairing ultra-high pressure liquid chromatography (UHPLC, Dionex-ThermoFisher) combined with a electrochemical (EC) detector (Coulochem III, ESA) under isocratic conditions (Shepherd et al., 2006). The guard cell was set at +350 mV, with a screening electrode set at −150 mV, and working electrode set at +220 mV. The mobile phase consisted of 75 mM sodium dihydrogen phosphate monohydrate, 1.7 mM 1-octane sulfonic acid sodium salt, 0.73 mM triethylamine, 25 μM EDTA, 10 % acetonitrile/90 % water. The pH was adjusted to 3.0 with phosphoric acid. The mobile phase was delivered at a flow rate of 0.6 ml/min onto the MD-150 (3 × 150 mm, 3 μm) reverse phase column (ESA, Chelmsford, MA, USA). Twenty microliters aliquots were injected by an autoinjector with cooling module set at 4 °C. The levels of DA, DOPAC, HVA, 3-MT in unknown samples were determined using standard curves created by injection of known concentrations of each compound.

2.2j) Statistical analysis

Comparison among more than two groups for experiments was analyzed by two-way ANOVA followed by the Bonferroni *post-hoc* test for *p* values. Comparison between just two groups was tested by the two-tailed Student's t-test. Specific stastitical tests and number of animals used in each group for every experiment are indicated in figure legends.

2.3) Results

2.3a) RGS10 expression in B cells, monocytes, and granulocytes is increased with age while microglial RG10 expression decreases

To assess whether RGS10 expression is important for regulation of immune cell subsets with aging, we first measured the level of expression in various immune cell subsets from the peripheral blood and spleen (**Fig 4**). In the peripheral blood, RGS10 expression significantly increases with age in CD19+CD45+ B cells, CD19-CD3-CD45+CD11b+Ly6G- monocytes, and CD19-CD3-CD45+CD11b+Ly6G+ granulocytes (**Fig 4A**). RGS10 expression did not increase with age in CD45+CD3+ T cells. In splenocytes, RGS10 expression significantly increases with age in monocytes, macrophages and granulocytes (**Fig 4B**). Then, we also quantified the RGS10 expression in Iba1+ cells in the brain by immunofluorescent staining demonstrating that the total level of RGS10 expression in Iba1+ cells, average level of RGS10 expression per Iba1+ cell, and average area of RGS10+ immunoreactivity per Iba1+ cell was decreased in aged mice (**Fig 4C**). Representative images of Iba1 and RGS10 staining visually confirm similar findings to quantification (**Fig 4D**). In summary, RGS10 expression in mice increases in B cells, monocytes, and granulocytes but decreases in Iba1+ cells in the brain with aging.

2.3b) Loss of RGS10 has minimal effect on frequency and number of peripheral immune cell subsets but does alter immune cell frequencies in the brain in young mice

To investigate whether loss of RGS10 alters the profile of immune cell subsets, we determined the frequency and number of immune cell subsets in the peripheral blood and spleen as well as the brain. There was a slight yet a significant decrease in the frequency of CD8+ splenocytes in RGS10-/- mice (**Fig 5A, 5B**). The frequency and number of other immune cell subsets in the periphery remained unchanged by loss of RGS10. In the brains of young RGS10-/- mice, the frequency of monocytes/microglia was decreased while the frequency of granulocytes and CD8+ T cells was increased. In addition, the number of granulocytes in the brains of young RGS10-/- was increased. Therefore, we see alterations in the immune cell repertoire of the brains of RGS10-/- young mice while the peripheral immune cell repertoire is not significantly altered.

2.3c) Loss of RGS10 alters B cell, M0, and CD4+ T cell frequency and number in the periphery of but not in the brains of aged mice

Since RGS10 expression increases with age in B cells, monocytes, and granulocytes, we predicted that the homeostatic frequencies of these cell types would be altered in aged RGS10-/- mice. Indeed in aged RGS10-/- mice, the frequency of B cells and CD4+ T cells in the peripheral blood was significantly decreased while the frequency of monocytes was significantly increased (**Fig 6**). Furthermore, the absolute number of monocytes and CD4+ T cells in spleens of aged RGS10-/- mice was decreased. In summary, the frequency and number of monocytes, B cells, and CD4+ T cells in the periphery but not in the brain was altered by the loss of RGS10 in aged mice.

2.3d) Loss of RGS10 does not alter serum cytokine levels, but is associated with loss of age-related increase in levels of IL-6 in the cerebrospinal fluid

Since loss of RGS10 in the context of aging altered the homeostatic frequency and number of immune cell subsets, we investigated whether loss of RGS10 altered serum or CSF cytokine or chemokine levels in this same context. Serum and cerebrospinal fluid from WT and RGS10-/- mice were collected and analyzed by Multiplex ELISA. There were no major differences in pro-inflammatory cytokine levels in young versus aged mice (**Fig 7**). The level of the chemokine, KC (CXCL1), increases with age but there is no difference between the genotypes. CSF was pooled within groups and analyzed on a multiplex ELISA. IL-6 level was increased by aging in WT mice but not in RGS10-/- mice. Level of TNF-α was increased with age in both genotypes in CSF. The levels of other cytokines in the multiplex ELISA were undetectable in the CSF in all groups. In summary, loss of RGS10 alters the dynamics of IL-6 in the CSF of mice but does not affect the levels of serum cytokines.

2.3e) RGS10 and Tyrosine Hydroxylase protein expression does not change with age in the ventral midbrain or striatum

Using midbrain dopaminergic cell lines in culture, we previously showed that in response to acute inflammatory insult, RGS10 levels were dramatically diminished [46]; however, it is unknown whether the events that occur during aging modulate RGS10 in nigrostriatal brain regions *in vivo*. To determine this, we measured RGS10 steady-state levels in ventral midbrain and striatum tissues from young and aged RGS10 WT mice. It was discovered that RGS10 protein levels were not altered with age in neither ventral

midbrain nor striatum tissues (**Fig 8A**). Next, to determine whether RGS10 is required for normal nigrostriatal DA neuron function in aging, we measured protein levels of the catecholamine neuron marker, tyrosine hydroxylase (TH), in young and aged RGS10 WT and-/- animals (**Fig 8B, C**). Interestingly, no differences in TH levels were noted in ventral midbrain tissues when comparing between RGS10 WT and-/- animals in young or aged groups (**Fig 8B, C**). This finding suggests that RGS10 gene ablation spares nigrostriatal DA neurons, even during aging.

2.3f) Loss of RGS10 does not alter tyrosine hydroxylase, Parkin, or Nrf2 mRNA expression in the ventral midbrain in young or aged mice

In efforts to confirm that aging does not alter the expression of RGS10 in nigrostriatal brain regions, RGS10 mRNA levels were quantified in young and aged animal cohorts using QPCR. As expected, no significant differences were noted when comparing RGS10 mRNA levels in RGS10 WT young and aged animal ventral midbrain or striatum tissues (**Fig 9A**). Further, also consistent with the observations made on the protein level, RGS10 gene ablation did not alter TH mRNA levels in mice, nor did advancing age (**Fig 9B**).

Next, since we have previously shown deficits in the Parkinson's disease implicated gene product, parkin, to be causal of inflammation-related dopaminergic neuron toxicity [193] , it was of interest to determine whether RGS10 may be implicated in this pathway. Thus, we measured mRNA levels of parkin in young and aged RGS10 WT and-/- animal ventral midbrain tissues. However, significant differences in the levels of parkin mRNA were not observed when comparing between any of the animal groups (**Fig 9B**).

It was also considered that inflammation is often correlated with an increased incidence of oxidative stress. Thus, it was hypothesized that if RGS10 acts to diminish neuroinflammation in DA neurons by modulating the effects of harmful oxidation. To begin to address this hypothesis, expression levels of the well-characterized antioxidant stress response transcription factor, Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), were compared in young and aged RGS10 WT and-/- animal ventral midbrain tissues. Interestingly, alterations in Nrf2 mRNA levels were not detected when comparing between any of the animal groups (**Fig 9B**). Together, this evidence indicates that the ablation of RGS10 alone may not be sufficient to induce deleterious effects in dopaminergic neurons, even during aging.

2.3g) Loss of RGS10 does not alter dopamine metabolism in the nigrostriatal pathway

Although it was observed that TH levels were not modulated by ablation of RGS10 in animals, it was of interest to determine whether loss of RGS10 alters the function of dopaminergic neurons in the nigrostriatal pathway. To assess this, striatal tissues from young and aged RGS10 WT and-/- animals were analyzed for the levels of DA and its metabolites using HPLC with electrochemical detection. Although no differences in the levels of DA were observed when comparing between any of the animal groups (**Fig 10**), interestingly, it was discovered that the degradation rate of DA in presynaptic dopaminergic neuron terminals was significantly decreased with age in RGS10 WT mice, as indicated by a ~1.25 fold lower ratio of striatal 3-4-dihydroxphenyl-acetic acid (DOPAC)/DA levels in comparison to the young RGS10 WT group. This effect was not seen in the RGS10-/- mice (Fig 10B, left panel). Notwithstanding, it is worth noting that

young RGS10-/- mice exhibited a trend towards diminished striatal DOPAC levels in comparison to the young RGS10 WT mouse group, though the results were not significant (data not shown). No significant differences in the ratios of homovanillic acid (HVA)/DA levels or 3-methoxytyramine (3-MT)/DA levels were observed when comparing between any of the mouse groups, suggesting that neither age nor loss of RGS10 significantly alter DA degradation that occurs in extranigral cell types.

2.4) Discussion

RGS10 plays a critical role in neuroimmune interactions through negative regulation of NF-kB signaling in microglia and interaction with the PKA/CREB pathway in dopaminergic neurons [29,46,47]. In previous studies, global loss of RGS10 in mice results in chronic microgliosis and loss of dopaminergic neurons in response to chronic peripheral administration of LPS [46]. Given this *in vivo* phenotype and because aging is the strongest risk factor for neurodegenerative disease [176,177], we sought to understand the role of RGS10 in DA neurons and immune cells in the context of aging. Loss of RGS10 does not significantly alter the distribution of peripheral immune cell subsets or dopaminergic neuron number in young mice (**Fig 5, 8-10**). In the brain, an increase in CD8+ T cell and granulocyte populations and a relative decrease in level of monocyte/microglia populations was seen in young RGS10-/- mice (**Fig 5C**). With age, RGS10 expression increases in B cells, monocytes, and granulocytes while decreasing in Iba1+ brain cells (**Fig 4**). As suggested from the dichotomous change in RGS10 expression between the periphery and brain in specific immune cells with age, we see a difference in the timing of dysregulation of the frequency and number of immune cells in

RGS10-/- mice between the periphery and the brain. We observed decreased frequencies of CD4+ T cells and B cells in the peripheral blood, increased frequency of peripheral blood monocytes, and decreased number of CD4+ T cells and monocytes/macrophages in the spleen (**Fig 6**). RGS10 seems to play a critical role in the regulation of immune cell profiles during aging. In young mice, loss of RGS10 alters the immune cell repertoire in the CNS while leaving the peripheral immune cell repertoire largely unaltered. This pattern is reversed in aged mice just as the level of RGS10 expression becomes reversed between the periphery and CNS. These changes may suggest that RGS10 may be important for the maintenance of immune cells at different times throughout the lifespan in different compartments. The changes seen in T cell populations, in which RGS10 expression does not increase with age, may be compensatory in nature to changes in number and frequency other immune cell types. While there is no dysregulation in immune cell populations in the brains of aged RGS10-/- mice, we observed an absence of increased levels of IL-6 in the CSF relative to WT mice. IL-6 is known to play an important role as a proinflammatory signal by inducing activation of immune cells and of hematopoeisis, production of acute phase reactants, and recruitment of immune cells to sites of inflammation [194]. It remains to be determined whether the loss of dynamics in IL-6 in CSF of aged RGS10-/- mice is in response to dysregulated immunity in the CNS (as seen in young mice) or a consequence of dysregulation of immune cell populations in the periphery in aged RGS10-/- mice. In summary, loss of RGS10 significantly impacts the immune cell repertoire of the young mouse brain and the aged mouse peripheral immune system.

In contrast, RGS10 does not seem to play a critical role in modulating the function of nigrostriatal dopaminergic neurons in aging as one would predict due to their sensitivity to peripheral inflammation in the RGS10-/- mouse. RGS10 levels are not altered in the ventral midbrain or striatum with age (**Fig 8**). Furthermore, proteins that are critical for dopaminergic neuron function are not altered by aging (**Fig 9**). Finally, nigrostriatal dopamine metabolism is not altered in an RGS10 dependent manner in mice (**Fig 10**). Taken together, these findings implicate RGS10 in regulation of immune cell populations in different compartments at different points of the lifespan rather than in dopaminergic neuron function. This dysregulated immune repertoire at different points in the lifespan is likely to indirectly affect neuron function and survival of dopaminergic and perhaps other neuronal populations and may in part explain the phenotypes that are seen in RGS10-/- mice and the human disease association with ARM.

In immune cells, RGS10 has been implicated in chemokine, SHP-1, NF-kB, and macrophage M1/M2 activation pathways [28-30,190]. RGS10 expression increased with age in B cells, granulocytes and monocytes while decreasing in microglia and the homeostatic frequency and number of these immune cell subsets were altered in RGS10-/- mice. These findings suggest that RGS10 could be playing important regulatory functions in B cells, granulocytes, microglia and monocytes at different points in the lifespan. Given that CD4+ T cell frequency and number is altered in aged RGS10-/- mice, RGS10 may also be playing a homeostatic role independent of changes in expression level in CD4+ T cells with age or these changes could be compensatory in response to changes in B cell and monocyte populations. Granulocyte expression of RGS10 increases with age but granulocyte populations were only altered in the brains of young RGS10-/-

mice. RGS10's roles in cellular activation in monocytes/macrophages [30] and chemotaxis in T cells [28] provide clues that activation signals and homing to lymphoid organs may be altered in RGS10-/- animals that leads to altered distribution of immune cell subsets throughout the lifespan. Lack of an increase in IL-6 in the CSF of aged RGS10-/- mice further suggests that dysregulated immune cell recruitment or activation may underlie observed changes. Future studies on RGS10 in aging should explore the precise immune-related mechanisms that drive the dyresgulation of immune cell population profiles.

One subtle but important point in this study is that we do not see increased levels of microglia/monocytes in the CNS of RGS10-/- mice on a pure genetic background unlike previously reported for RGS10-/- mice on a mixed genetic background [46]. Specifically, the first studies where chronic microgliosis was observed in RGS10-/- mice was performed on mice of mixed genetic background (129/C57/BL6). All other subsequent studies from our group including the studies reported herein were performed on RGS10-/- mice on a pure genetic background (C57/BL6). Unsurprisingly, genetic background and environment can have variable impact on phenotype. While we do not see chronic microgliosis on the pure background strain of RGS10-/- mice, there is robust and reproducible *in vivo* immune dyregulation and *ex vivo* studies of microglia/macrophages support this conclusion [29,30,47].

Overall, our data indicates that RGS10 has a role in modulating the regulation of immune cells throughout lifespan. Further understanding of the precise role of RGS10 in these immune cell populations will enhance our understanding of how dysregulated immune processes may increase the risk for age-related neurodegenerative disease. Specifically, understanding the role of altered immune function will be important for

developing novel therapies for neurodegenerative disease that show a strong inflammatory component such as Parkinson's disease and age-related maculopathy [77,177]. The role of RGS10 in human disease has not been thoroughly assessed but age-related maculopathy pathology demonstrates significant microglial activation [41]. Interestingly, RGS10 expression in the mouse cornea increases with age but this change in expression has not be contextualized in human disease [195]. Extended immunophenotyping studies of human peripheral blood and post-mortem brains are warranted to elucidate whether altered expression of RGS10 in immune cells is detectable in age-related diseases characterized by inflammation such as age-related maculopathy and neurodegenerative diseases like Parkinson's disease.

2.5) Figures

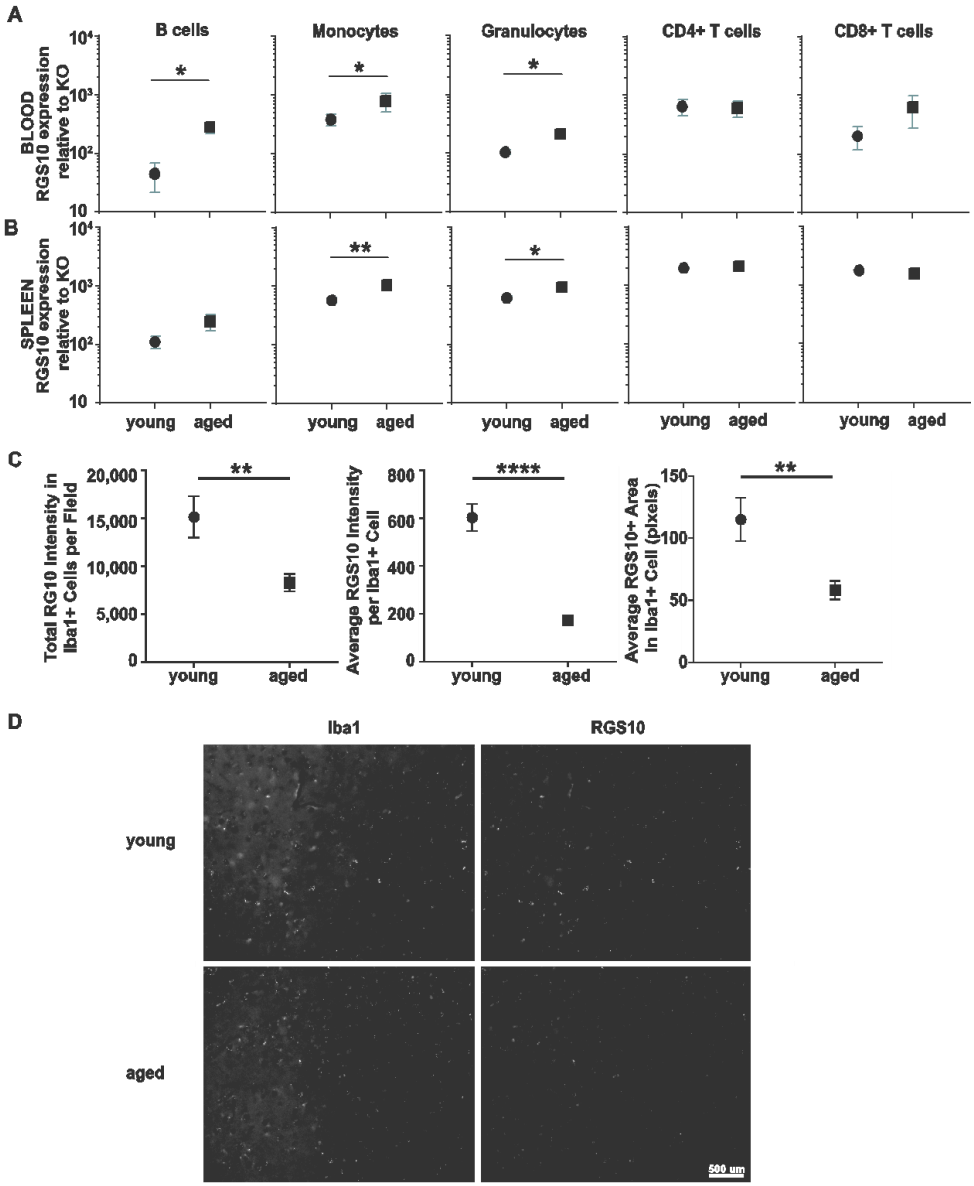

Figure 4. RGS10 expression increases in peripheral B cells, monocytes/macrophages, and granulocytes and decreases in Iba1+ cells in the brain with age. RGS10 expression was measured by intracellular staining for flow cytometry in B cells, monocytes/macrophages, granulocytes, and T cells from **A)** peripheral blood and **B)** spleens of young (circles) and aged (squares) mice, n = 4-5/group. **C)** Total RGS10 intensity in Iba1+ cells and average RGS10 intensity and area per Iba1+ cell in 20

fields per mouse (n=3 per group). **D)** Representative images of RGS10 and Iba1 staining from brains of young and old mice. Data are plotted as mean +/- standard error of the mean (SEM) for each group. P-values are indicated for two-tailed t-test: * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$.

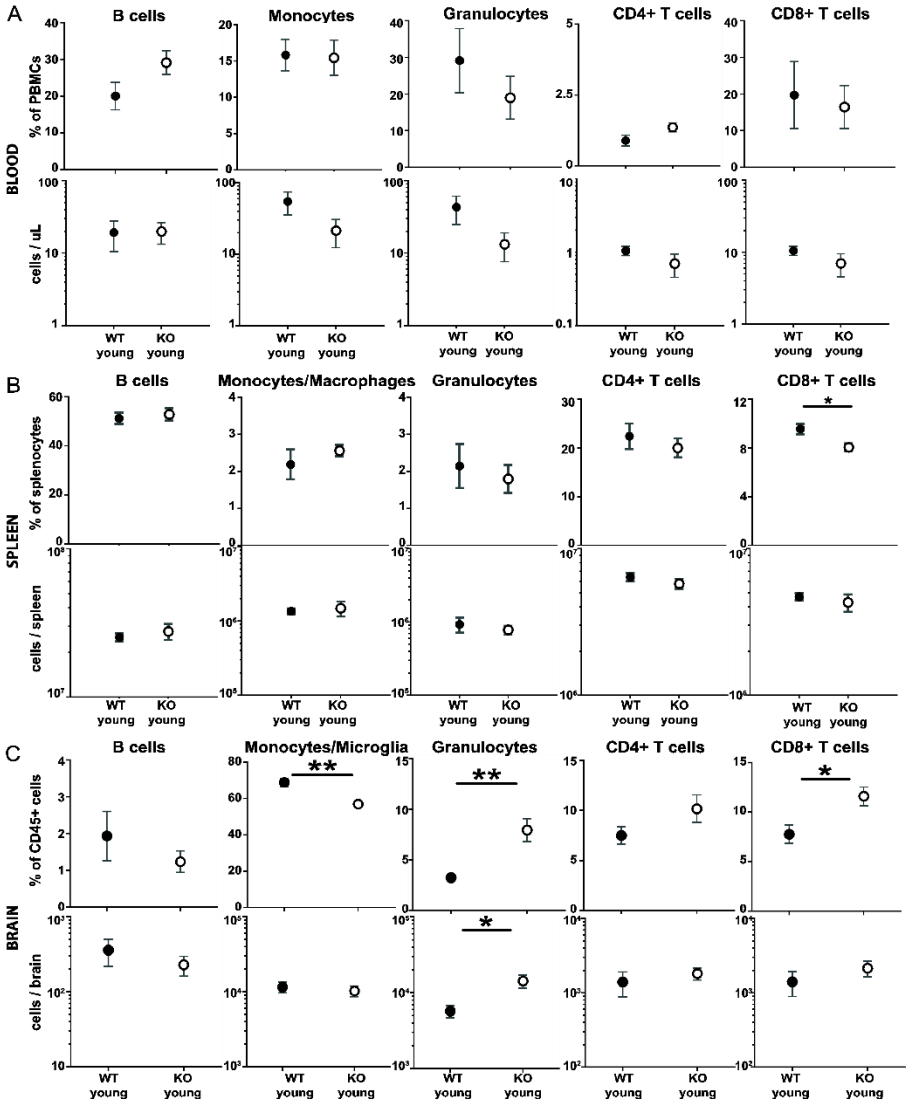

Figure 5. Loss of RGS10 alters the immune cell repertoire in the brain but has little effect on immune cell frequency and number in the periphery of young mice.

Immune cell frequency and number was measured by flow cytometry in **A)** peripheral

blood, **B)** spleens, and **C)** brains of young WT (black) and RGS10-/- (white) mice, n = 7-9 per group. Data are plotted as mean +/- SEM for each group. P-values are indicated for two-tailed t-test: * $p < 0.05$.

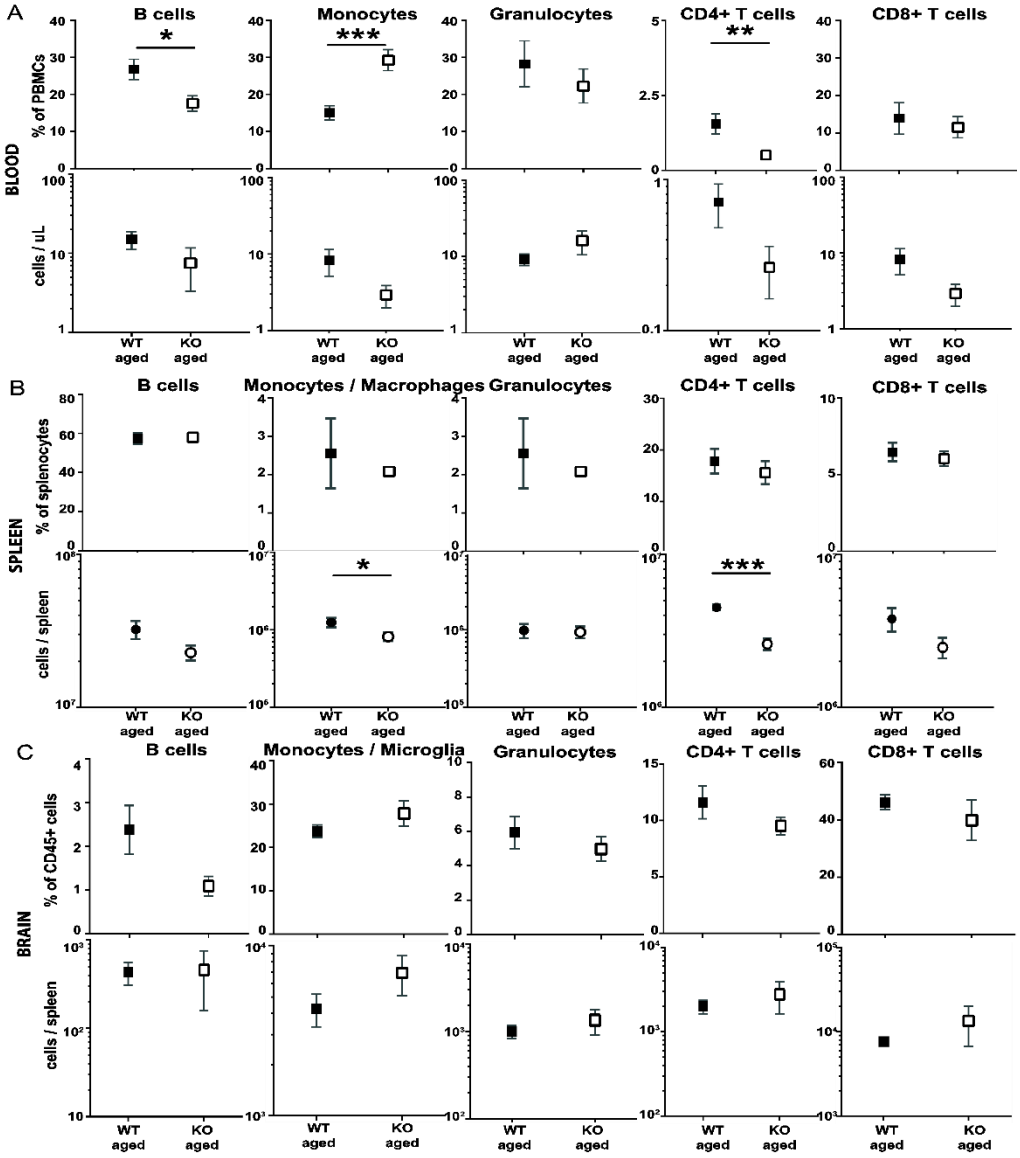

Figure 6. Loss of RGS10 alters B cell, monocyte/macrophage, and CD4+ T cell frequency and number in the periphery but not in the brain of aged mice. Immune cell frequency and number was measured by flow cytometry in **A)** peripheral blood, **B)** spleens, and **C)** brains of aged WT (black) and RGS10-/- (white) mice, n = 10-13 per

group. Data are plotted as mean +/- SEM for each group. P-values are indicated for two-tailed t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

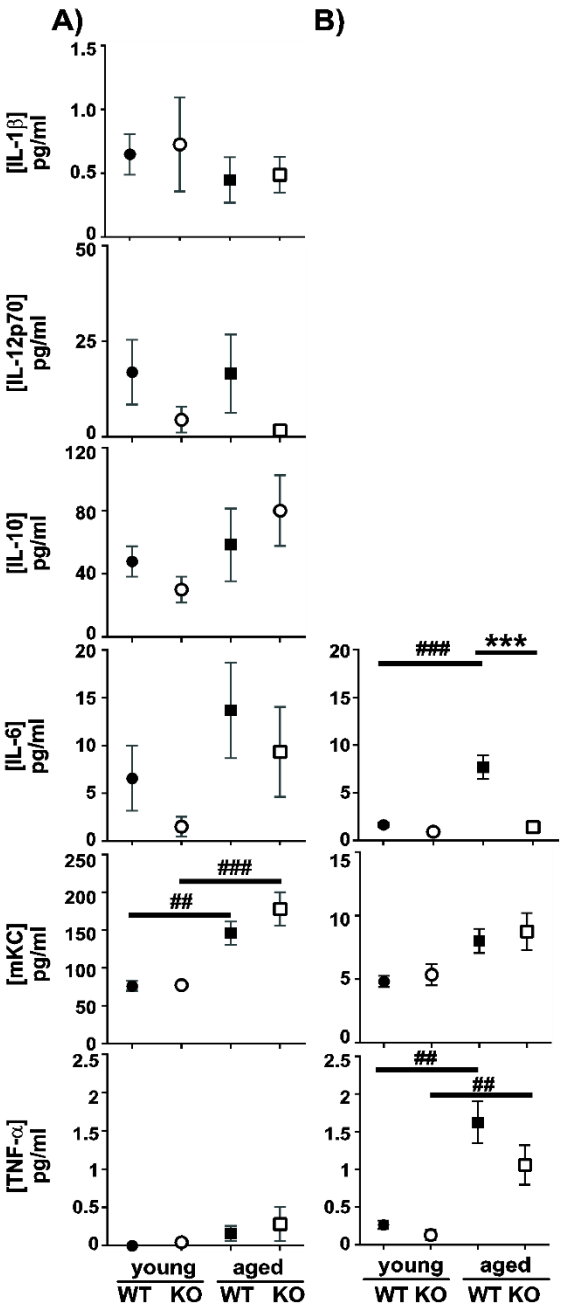

Figure 7. Loss of RGS10 does not alter serum cytokine levels but decreases IL-6 levels in the CSF in aged mice. Cytokine levels in **A)** serum and **B)** CSF from young (circles) and aged (squares) WT (black) or RGS10-/- (white) mice were measured using

7-plex MesoScale Discovery Assay Kit. Serum was collected from n=9-10 mice/group and data are plotted as mean +/- SEM for each group. CSF was pooled within groups (n = 3 pools of 3-4 mice in each group). Therefore, pooled samples from 9-12 animals were analyzed. P-values are indicated for one-way ANOVA with post-hoc Holm-Sidak test. *** $p < 0.001$ for genotype-specific changes and ## $p < 0.01$, ### $p < 0.001$ for age-specific changes.

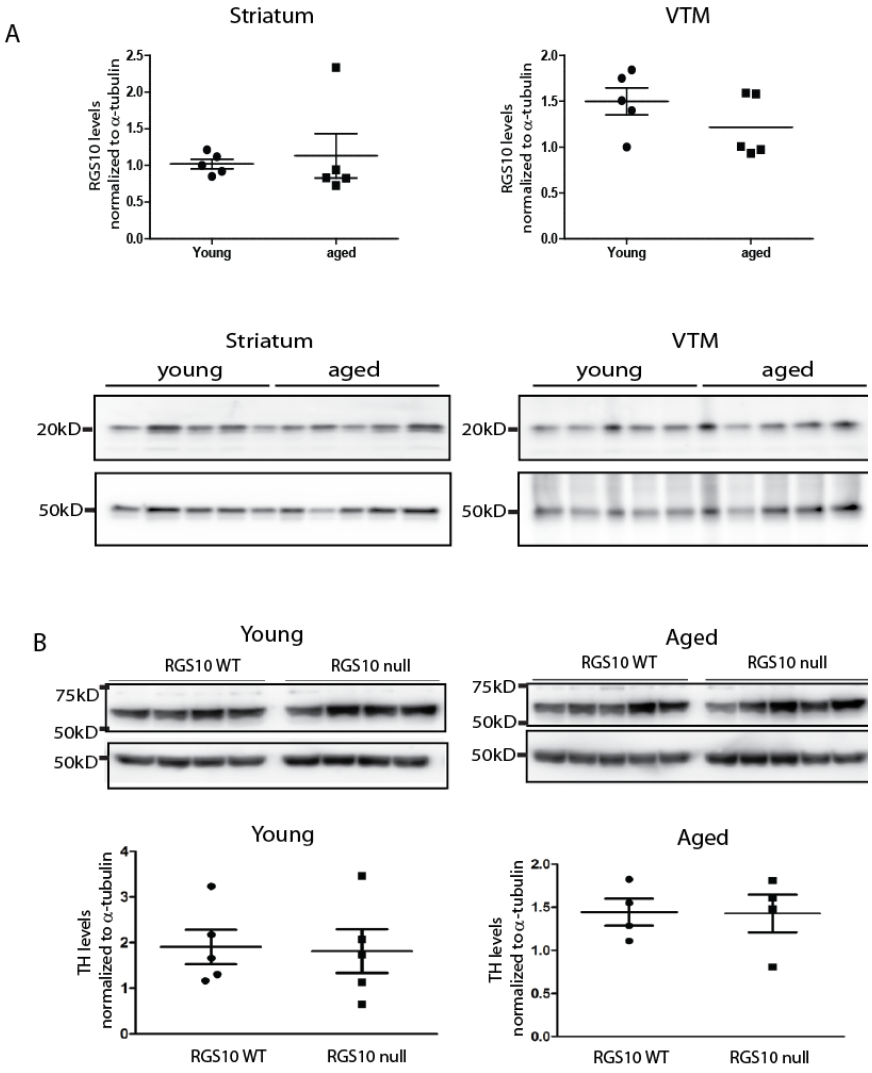

Figure 8. RGS10 expression does not change with age in the ventral midbrain or striatum and does not alter TH expression in the ventral midbrain in young or aged

mice. A) Soluble protein lysates from ventral midbrain (VMB) or striatal tissues of young and aged RGS10 WT animals were analyzed for the levels of RGS10 protein expression. B) Total protein lysates were extracted from VMB tissues of young and aged animals and were assessed by immunoblot for the levels of TH and α-tubulin. Membranes were also probed with mouse monoclonal α-tubulin antibody to assess protein loading. RGS10 expression was quantified by densitometry and normalized to α-tubulin expression. Data were plotted as a scatter with each point representing an individual animal and mean +/- SEM for each group. Two-tailed student's t-test was used to test for significance. No significant differences were noted.

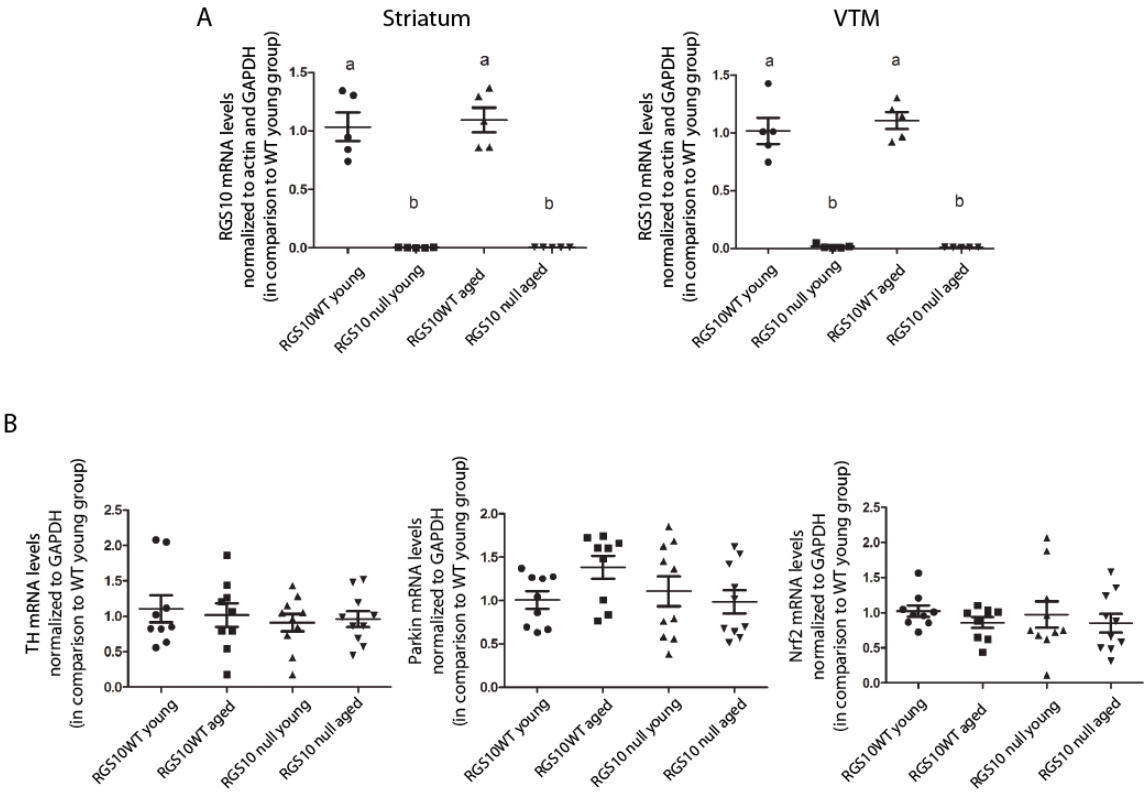

Figure 9. Loss of RGS10 does not alter mRNA expression for TH, Parkin, or Nrf2 in the ventral midbrain. A) Total RNA was extracted from ventral midbrain (VMB) or striatal tissues of young or aged RGS10 WT or RGS10-/- animals and was analyzed by

qRT-PCR. The resulting Ct values were analyzed by the ΔΔCt method using the RGS10 young wild-type group as the control group and the mean of Ct values for actin and GAPDH. Experiments were performed in triplicate. Data were plotted as a scatter with each point representing an individual animal and mean +/- SEM for each group. Data was analyzed by one-way ANOVA followed by bonferroni's post-hoc test. Columns with matching lower case letters are not statistically different. $p<0.0001$ **B**) VMB tissues from young and aged animals were analyzed by QPCR using primers directed against murine TH, Parkin, Nrf2, and GAPDH genes. For each animal, the Ct values for TH, Parkin, and Nrf2 were normalized to the Ct values for GAPDH. The data was graph and analyzed as previously described. No statistically significant differences were observed.

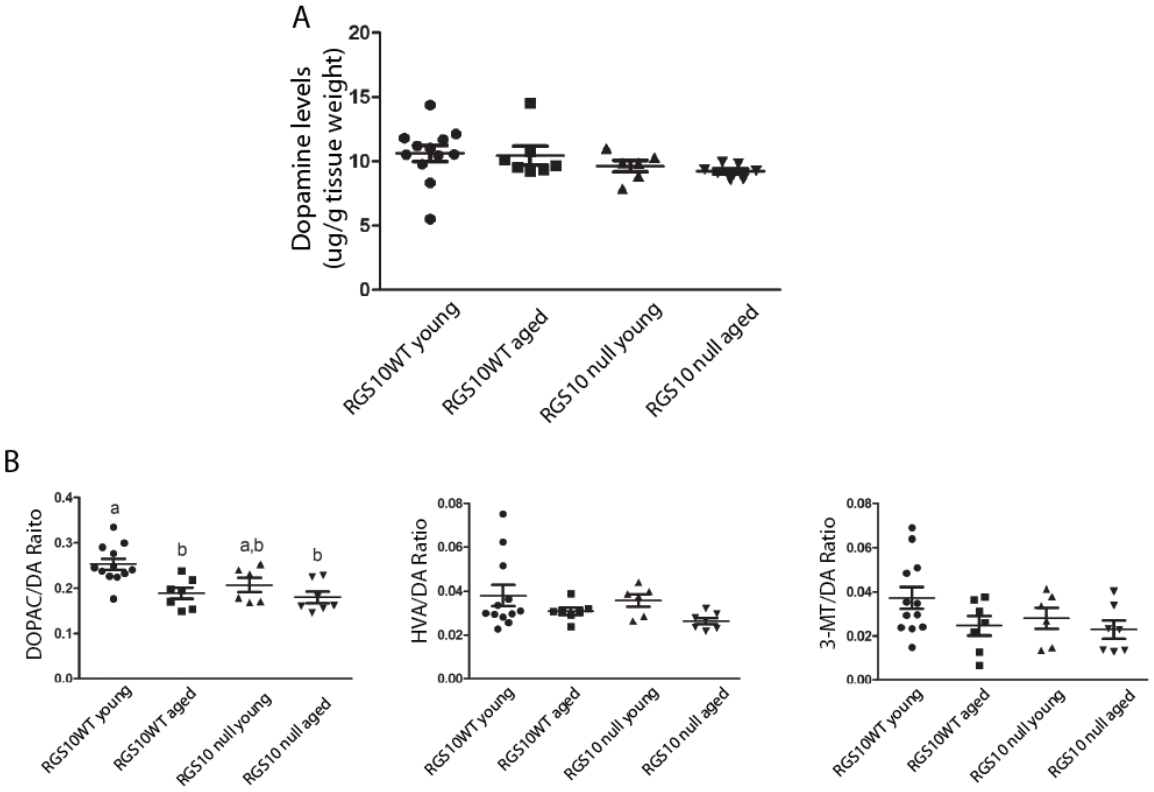

Figure 10. The levels of nigrostriatal DA and metabolites are independent of RGS10 expression. Coronal sections of striatum (2mm) were dissected from young and aged

RGS10 WT and-/- mice (n = 4 per age group, per genotype). Tissue was weighed and homogenized in 10 volumes of ice-cold catechol extraction buffer. Following sedimentation, the resulting supernatants were filtered and filtrates were analyzed using reverse-phase ion pairing ultra-high pressure liquid chromatography combined with an electrochemical detector under isocratic conditions. Analyte levels were normalized to tissue weight. In **A**), the micrograms of dopamine (DA) per gram of tissue are shown for the respective animals groups. In the scatter plots, the horizontal line denotes the mean of the indicated group. The error bars indicate the standard error of the mean. Each point represents a distinct animal. The graph was analyzed by one-way ANOVA and Bonferroni's post hoc test. No significant differences were observed. **B**) The ratio of the levels of DA metabolites 3-4-dihydroxphenylacetic acid ("DOPAC"), homovanillic acid ("HVA"), or 3-methoxytyramine ("3-MT") to DA were calculated for each animal and graphed. Graphs were analyzed by one-way ANOVA and Bonferroni's post hoc test. Columns with matching letters are not statistically different. $p<0.01$

Chapter 3: Regulator of G-Protein Signaling 10 modulates immune cell chemotaxis in neuroinflammation

3.1) Introduction

Regulator of G Protein Signaling (RGS) 10 modulates interactions between the immune and nervous systems, making it an interesting target to study in the context of neuroinflammation[179]. For human disease, the RGS10 locus has been associated with both schizophrenia and age-related maculopathy, degeneration of the retina associated with significant microgliosis[42,43,180]. The RGS10-/- mouse phenotype seems to support a dysregulated neuroinflammatory response that would be in line with this locus' disease associations. Specifically, the RGS10-/- mouse has chronic microgliosis and susceptibility to degeneration of midbrain DA neurons with administration of peripheral low dose lipopolysaccharide[46,47]. RGS10 has been implicated in numerous immune signaling pathways but no specific G-protein regulated pathway has been identified as its physiologic target.

RGS10 is the smallest of the RGS proteins and is highly expressed in the brain, thymus, and lymph nodes[3,25,31,181]. In heterologous assays it is known to selectively accelerate the GTPase activity of $G\alpha_{i3,}$ $G\alpha_{q,}$ $G\alpha_{z}$[25]. G-protein coupled receptors (GPCRs) control critical cellular and physiological functions through heterotrimeric G-proteins that are made up of an α subunit and a βγ heterodimer[23,182,183]. In immune cells, there are many G-proteins that are downstream of antigen-specific receptors as well as chemokine receptors[7,9,10]. RGS10 has been implicated in immune cell function through numerous studies[28,30,38,45]. Specifically, RGS10 negatively regulates NF-κB signaling, explaining the pro-inflammatory phenotype of RGS10-/- microglia and macrophages[30,47]. In

platelets, RGS10 negatively regulates platelet activation and binds to SHP-1, a known negative regulator of immune activation[190]. In addition, in aged mice, the levels of RGS10 increase in peripheral immune cells but decrease in CNS immune cells[175]. These changes in RGS10 levels in immune cells with age were associated with changes in relative levels of immune cells in the peripheral and central compartments suggesting a critical role for immune cells throughout the lifespan[175].

Understanding the critical pathways that RGS10 physiologically regulates will allow for development of targeted therapies for neuroinflammatory disorders. Other RGS proteins have been implicated in regulating chemotaxis[4,12,13,28]. In human T cells, RGS10 was shown to negatively regulate responses downstream of CXCL12 stimulation including integrin activation, adhesion to endothelium under shear stress, and chemotaxis[28]. RGS10 was shown to regulate this pathway via opposition of activation of the Vav-Rac pathway downstream of CXCL12 stimulation[28]. Dysregulation of immune cell chemokine signaling could explain the phenotypes we see in the RGS10-/- mouse. To test this idea, we hypothesize that lack of RGS10 will lead to increased chemokine-induced cellular responses such as integrin activation, adhesion, and chemotaxis that in turn lead to altered in vivo immune cell trafficking.

3.2) Materials and Methods

3.2a) Animals

Generation of RGS10-/- mice (C57/B6) has been described previously [47]. Two to three month old male and female mice were used for experiments. Experimental procedures involving use of animal tissue were performed in accordance with the NIH Guidelines for Animal Care and Use and approved by the Institutional Animal Care and Use Committee

at Emory University School of Medicine in Atlanta, GA. Mice were euthanized by intraperitoneal Euthasol injection.

3.2b) Flow Cytometry

From mice, spleens were homogenized into a single-cell suspension and blood was collected in EDTA-coated tubes by cheek bleed and then red blood cells (RBCs) were lysed with RBC lysis buffer (1.5M NH_4Cl, 0.1 M $KHCO_3$, Na_2EDTA, pH 7.4). Staining for dead cell exclusion was performed on ice for 30 minutes using Fixable Red or Aqua dyes (Life Technologies). For surface staining, cells were washed with FACS buffer and then stained for 20 minutes with fluorophore-conjugated antibodies. For mouse tissues, the antibodies used were anti-CD11b-FITC (ebiosciences), anti-CD19-PE (ebiosciences), anti-CD3e-PE-Texas Red (ebiosciences), anti-MHC-II-PerCP-e710 (ebiosciences), anti-CD45-PerCP-Cy5.5 (ebiosciences), anti-NK1.1-PE-Cy7 (ebiosciences), anti-CD45-APC (ebiosciences), anti-Ly6C-APC-Cy7 (ebiosciences), anti-Ly6G V450 (BD Horizon), anti-CD11c AF700 (ebiosciences), and anti-CD16/CD32 (ebiosciences). For human tissue, the antibodies used were anti-CD14-PE (Biolegend), anti-CD19-PE-Cy7 (Biolegend), anti-HLA-DQ-APC (Biolegend), anti-CD8-APC-Cy7 (BD Biosciences), anti-CD3-e450 (ebiosciences), anti-HLA-DR-V500 (Biolegend), anti-CD4-BV650 (Biolegend), Human TruStain FcX Receptor Blocker (Biolegend), and anti-CD16-Alexa Fluor 700 (Biolegend). If applicable, intracellular staining was then performed using Invitrogen Fixation and Permeabilization Media with goat anti-RGS10 primary antibody (Santa Cruz Biotechnology) and donkey anti-goat IgG PerCP-Cy5.5-conjugated secondary antibody (Santa Cruz Biotechnology). If intracellular staining was

not performed, cells were washed and then fixed with 1% paraformaldehyde for 30 minutes. After washing, cells were stored in FACS buffer until analysis on a LSR-II flow cytometer (BD Biosciences). Data analysis was performed on FlowJo software.

3.2c) Immune Cell Isolation from Adult Mouse Brain

Microglia were isolated from adult mice as described previously [191] . Briefly, brain was finely minced using a scalpel and digested using a papain-dispase solution at 37°C. Microglia and other immune cells were separated from myelin, red blood cells, and other cell debris by collecting the 30:37 layer interface of a 30:37:70 Percoll gradient. Immune cells were washed out from Percoll with 1x Hank's Balanced Salt Solution by density centrifugation.

3.2d) Boyden Chamber Assay for Chemotaxis

All chemokines used were diluted to 100 nM in RPMI-1640 (Corning) with 10% fetal bovine serum (Sigma) and 600 uL of total volume added to each bottom chamber of well. Cells were plated at a concentration of 0.5 million cells in 100 uL of RPMI-1640 with 10% fetal bovine serum in upper chamber of transwell with 5 um pore in a well of a 24 well plate (Costar). An "input" control well was also added to control for variability in pipetting between wells. Plate was incubated for 2 hours at 37°C upon which contents of bottom chamber were collected and analyzed by flow cytometry. CCL2, CCL5, CX3CL1, CCL19, and CXCL12 were purchased from Biolegend while sphingosine-1-phosphate (S1P) was purchased from Cayman Chemicals.

3.2e) Intracranial Administration of LPS/IFN-γ

For duration of surgery, animals were anesthetized with vaporized isoflurane (Baxter) and 0.3 mg/kg buprenex (Reckitt Benckiser) was given for analgesia 20 minutes prior to recovery from anesthesia. Intracranial injection of 5 uL of 0.9% saline (Braun) or 100 ng/mL lipopolysaccharide (LPS, Sigma) and 10 U/mL IFN-γ (Biolegend) was performed stereotaxically (AP: -0.2 mm ML: -1.0 mm DV: -2.5 mm from dura) into the lateral ventricle at a rate of 0.5 uL/min. Needle was allowed to rest intracranially for 5 minutes before removal to ejection of solution was complete. Incision site was closed with 7 mm steel wound clips (Autoclip). After 24h, mice were euthanized for harvesting of immune cells of brain for flow cytometry analysis.

3.2f) Thioglycollate-induced Peritonitis Model

Peritonitis was induced by intraperitoneal injection of 1 mL of Thioglycollate broth (BD Biosciences). Injection of 1 mL of phosphate-buffered saline was used as a control. Animals were euthanized after 1 or 3 days and peritoneal lavage was performed to collect cells. The cells from the lavage fluid were analyzed by flow cytometry.

3.2g) Human Subject Recruitment

PD patients and age-matched healthy CTRL subjects were recruited through the Clinical Research in Neurology (CRIN) IRB-approved research protocol at the Emory Movement Disorders Clinic. Participants were excluded if they were younger than 50 years old, were older than 85 years old, or had neurologic, chronic infectious, or autoimmune comorbidities, and/or known familial PD mutations.

3.2h) Isolation of Human Peripheral Blood Mononuclear Cells

PBMCs were isolated from whole blood using Ficoll-Paque (GE Healthcare) density centrifugation. Upon isolation of the middle PBMC layers, cells were immediately stained for analysis by flow cytometry.

3.2i) Statistical analysis

Comparison among more than two groups for experiments was analyzed by two-way ANOVA followed by the Sidak *post-hoc* test for *p* values. Comparison between just two groups was tested by the two-tailed Student's t-test. Specific stastitical tests and number of animals used in each group for every experiment are indicated in figure legends.

3.3) Results

3.3a) Lack of RGS10 alters chemotaxis in a cell-specific and chemokine-dependent manner in mouse PBMCs

To test whether RGS10 regulates chemotaxis downstream of relevant chemokine pathways in mouse PBMCs, we performed a Boyden chamber assay with freshly isolated cells. Chemokines were chosen based upon relevance to cell types that have shown alterations in RGS10-/- mice, i.e. CCL2, CCL5, and CX3CL1 for myeloid cells and CCL19, CXCL12, and S1P for lymphocytes[28,30,175]. All cell types except CD3+ T cells from RGS10-/- animals had significantly decreased chemotaxis in response to CCL2 (**Fig 11**). Significantly enhanced chemotaxis of NK cells (CD45+CD3-CD19-NK1.1+) but dampened chemotaxis of granulocytes (CD45+CD3-CD19-NK1.1-CD11b+Ly6G+) was seen in cells from RGS10-/- animals in response to CCL5. Significantly increased

chemotaxis of RGS10-/- T cells was seen in response to CCL19. No significant differences were seen in chemotactic responses to CXCL12 and in response to CX3CL1, only granulocyte chemotaxis was significantly dampened in cells from RGS10-/- animals.

S1P normally recruits cells to secondary lymphoid tissues from the blood and then allows for the egress of immune cells from these lymphoid tissues a few days after activation[196]. To model these dynamics *in vitro*, responses to S1P of both freshly isolated PBMCs as well as unstimulated and 10 ng/mL phorbol myristate acetate (PMA), 0.5 uM ionomycin-stimulated splenocytes were assessed. There were no differences in PBMC recruitment to S1P (data not shown) but with *in vitro* stimulated splenocytes, increased chemotaxis of RGS10-/- dendritic cells was observed. In summary, RGS10-/- cells demonstrated chemokine and cell-type specific alterations in chemotaxis.

3.3b) Loss of RGS10 alters immune cell recruitment to in vivo models of inflammation

To assess immune cell chemotaxis *in vivo*, a peripheral model of inflammation as well as a model of brain inflammation was used. In the peripheral model of thioglycollate-induced peritonitis, we observed a significant decrease in the number and frequency of only CD11b+CD11c- non-granulocytic cells (CD45+CD3-CD19-NK1.1-Ly6G-) in the peritoneal lavage fluid of RGS10-/- animals after 3 days suggesting a subtle change in cellular recruitment (**Fig 12**). There was minimal observed recruitment of T cells, B cells, or NK cells in this model (data not shown). No changes were seen between RGS10 WT and -/- animals 1 day after induction of peritonitis (data not shown)

but as expected for this model, an increase in granulocytes was present in all animals that disappeared by day 3 when a large recruitment of other myeloid cells such as macrophages and dendritic cells is observed[197].

In the model of brain inflammation, LPS/IFNγ was acutely injected into the lateral ventricle. After 24 hours, robust recruitment of CD45-high cells has been previously reported[198]. Thus, we used this model to assess whether RGS10-/- mice had altered recruitment of immune cells to the brain. It was determined that RGS10-/- animals had no deficiency in recruitment of most immune cells to the brain but rather the CD45-low myeloid cell population did not increase as it did in the WT animals (**Fig 13**). This lack of increase in the CD45-low myeloid population explains why there is no increase in number of microglia/macrophages in the brain in RGS10-/- animals after LPS/IFNγ injection. Taken together, these results show that acute recruitment of immune cells in a peripheral and CNS model of inflammation is not severely impaired in RGS10-/- animals. Rather, a selective defect in recruitment of CD11b+CD11c- non-granulocytic myeloid cells to the peritoneal cavity and in expansion of the CD45-low myeloid cell population in the brain was demonstrated in the RGS10-/- mouse.

3.3c) Age-dependent decreases in RGS10 expression are present in CD8+ T cells and CD16+ monocytes from Parkinson's disease patients

Despite the phenotypes observed in RGS10-/- mice and from *ex vivo* stimulation of human cells, it is unclear whether altered RGS10 function plays a role in human disease[28,30,36,45,175]. To implicate RGS10 as a regulator of human immune cell function in

a neurodegenerative disease with chronic inflammation, immunophenotyping of aged healthy controls and Parkinson's disease patients was undertaken[1,77,86,199,200]. RGS10 expression is known to increase in peripheral immune cells in mice with age[175]. However, the characterization of RG10 in human immune cells has never been done. It was demonstrated that in PD patients, RGS10 levels decreased with age in CD8+ T cells and CD16+ monocytes (**Fig 14**). Furthermore, in CD16+ monocytes, there was a trend towards a decrease in RGS10 expression in PD patients relative to healthy controls ($p=0.57$). The results illustrate that in the context of a neurodegenerative disease, there are age and disease-related decreases in RGS10 expression.

3.4) Discussion

In this study, we set out to implicate RGS10 as a regulator of chemokine-dependent responses. Indeed, we demonstrated that RGS10-/- mouse PBMCs had altered chemotaxis in a cell-type specific and chemokine-dependent manner (**Fig 11**). The loss of RGS10 seemed to impact immune cell chemotaxis to CCL2 almost globally, while granulocyte chemotaxis in general seemed to be most altered by loss of RGS10 in response to numerous chemokines. Indeed, it is surprising that loss of a negative regulator would lead to decreased chemotaxis but these results may suggest that in the CCL2 pathway, RGS10 may be acting in a complex manner or have a non-canonical function. The increased chemotaxis of RGS10-/- T cells and DCs to CCL19 and S1P, respectively, could be relevant for trafficking to and from lymphoid organs. To elucidate the chemokine pathways that are directly regulated by RGS10, assessment of other downstream effector functions such as integrin activation and adhesion, as well as biochemical measurement of chemokine signaling are needed.

Given the altered measurements of *in vitro* chemotaxis in RGS10-/- cells, it was unexpected that RGS10-/- animals showed only subtle alterations in immune cell recruitment to acute models of inflammation. These subtle alterations could be a result of the promiscuous nature of chemokines which could allow for compensation of altered function in the *in vivo* setting[7]. In thioglycollate-induced peritonitis, only the recruitment of CD11b+CD11c-Ly6G- myeloid cells was dampened in RGS10-/- cells (**Fig 12**). This population of myeloid cells is often characterized as a macrophage population[201]. This result is consistent with *in vitro* Boyden chamber assays because CCL2-mediated chemotaxis was severely impaired in Ly6C+ monocytes which migrate to sites of inflammation and differentiate into macrophages. But unlike what the *in vitro* assay would have predicted, there was no difference in granulocyte recruitment to the peritoneal cavity in this model (data not shown) which could be a result of a compensatory effect in that cell type. In an acute model of CNS inflammation, immune cell recruitment was not altered in RGS10-/- mice but expansion of the CD45-low myeloid population was dampened (**Fig 13**). The CD45-low population is often characterized as mostly microglia suggesting that this result could implicate a defect in microglial proliferation rather than immune cell chemotaxis[104,198]. Clarification of the biochemical pathways downstream of chemokine signaling that RGS10 directly regulates may inform which inflammatory models would be more severely impacted in RGS10-/- mice. For example, a more chronic inflammatory model may show more interesting phenotypes given the increased duration and lower level of immune signaling that occurs.

To contextualize this work in human disease, we assessed the changes in RGS10 expression between the immune cells of healthy controls and PD patients. There was a

trend to decreased RGS10 expression in CD16+ monocytes in PD patients, the monocyte subset that readily migrates to sites of active inflammation (**Fig 14**)[202]. These findings fit well with the dopaminergic neuron susceptibility in RGS10-/- mice to peripheral LPS administration. Furthermore, there was an age-dependent decrease in RGS10 expression in CD8+ T cells and CD16+ monocytes in PD patients that may be related to disease progression or severity. In aged mice, RGS10 expression is increased relative to young mice in granulocytes, B cells, and monocytes[175]. It remains to be seen whether this age-dependent increase in humans occurs. Within the context of disease, loss of RGS10 expression could result in dysregulation of immune cell function. Given these initial studies linking RGS10 to regulating chemotaxis and previously published work on affecting immune cell function, changes in RSG10 expression could help explain dysregulation of inflammation in diseases like PD. Chemotaxis' role in altering localization and survival of immune cells could have a systemic impact on the immunoregulation in an organism explaining dysregulation of immune cells subsets in the mouse with aging and in the human with PD[4,7,175]. Understanding how RGS10 plays a role in neuroinflammation through its regulation of chemokine signaling could lead to its targeting as a target for PD and other neuroinflammatory conditions.

3.5) Figures

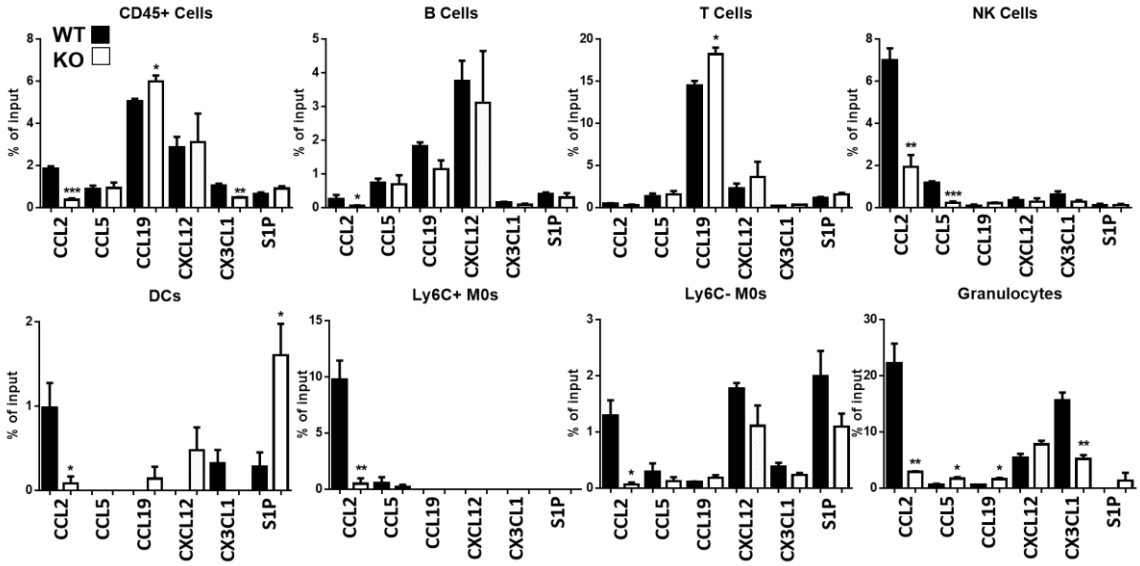

Figure 11. The loss of RGS10 alters immune cell chemotaxis in a cell-type specific manner to many immunologically relevant chemokines. Peripheral blood mononuclear cells were isolated from 2-3 month old male and female WT (filled) or RGS10-/- (open) mice (n=5/group). For S1P, splenocytes were isolated and stimulated for 3 days with 10 ng/mL PMA and 0.5 uM ionomycin. Cells were pooled within genotype group and placed in triplicate in a Boyden chamber with chemokines at 10nM in the bottom chamber. Number and type of immune cell migrated to the bottom chamber was assessed after 2 hours using flow cytometry. Student's t-tests were performed between WT and -/- groups within each condition. Significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data is plotted as mean +/- SEM.

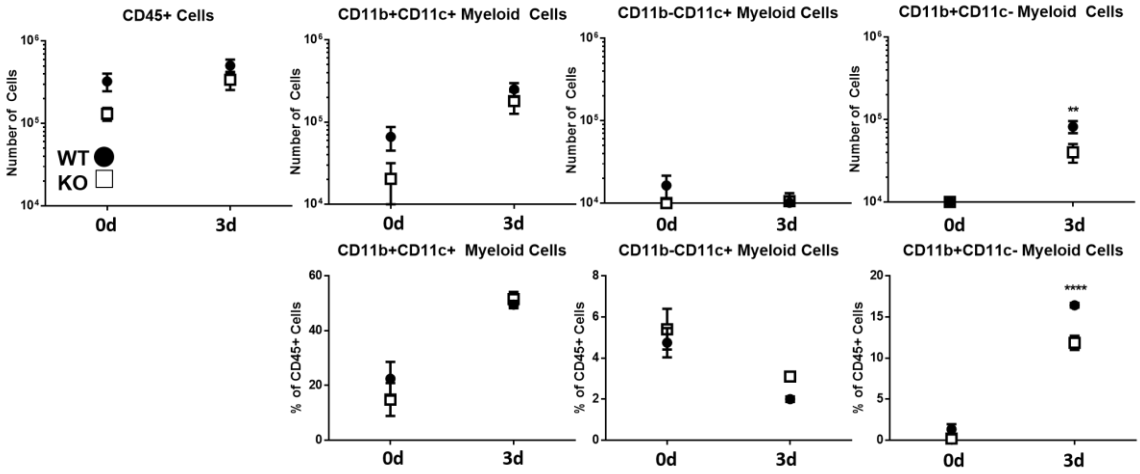

Figure 12. The loss of RGS10 increases the recruitment of CD11b+CD11c- myeloid cells 3 days after induction of thioglycollate-induced peritonitis. Two to three month old male and female WT (filled) or RGS10-/- (open) mice (n=5-6/group) were injected intraperitoneally with 1mL of thioglycollate broth. After 3 days, number and type of recruited cells were assessed by flow cytometric analysis of peritoneal lavage fluid. A two-way ANOVA followed by Sidak's post-hoc multiple comparisons test was used to assess differences between WT and -/- cells at each time point. Significance is indicated as ** $p < 0.01$, **** $p < 0.0001$. Data is plotted as mean +/- SEM.

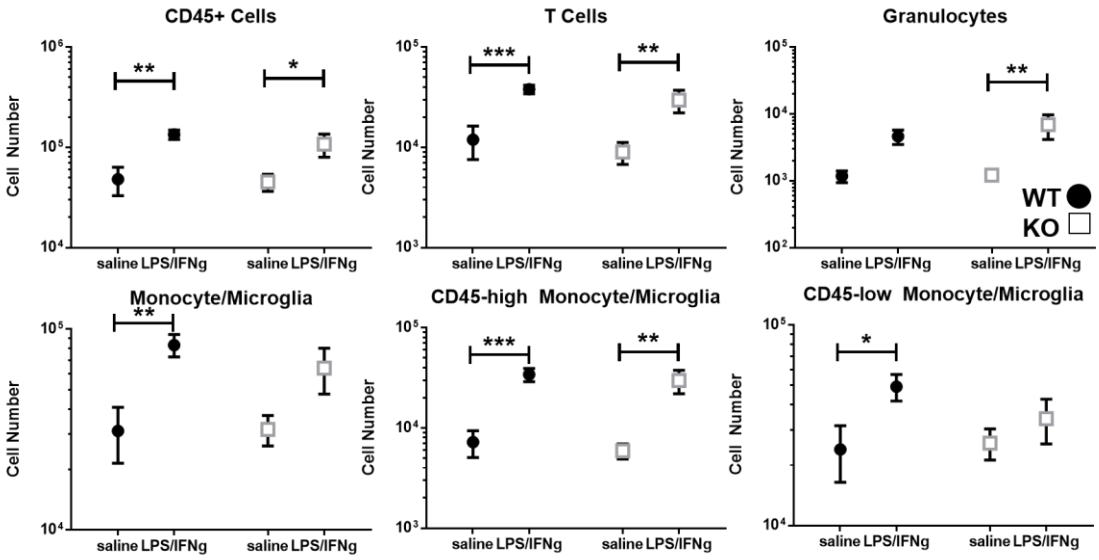

Figure 13. The loss of RGS10 dampens the increase in CD45-low monocyte/microglia number in response to intracranial LPS/IFNγ. Two to three month old male and female WT or RGS10-/- mice (n=5-6/group) were injected intraventricularly with 5uL of 100ng/mL LPS and 10 U/mL IFN-γ. After 24 hours, number and type of recruited cells were assessed by flow cytometric analysis of brain homogenate. A two-way ANOVA followed by Sidak's post-hoc multiple comparisons test was used to assess differences between WT (filled) and -/- (open) cells in saline versus LPS/IFNγ injected brains. Significance is indicated as ** $p < 0.01$, **** $p < 0.0001$. Data is plotted as mean +/- SEM.

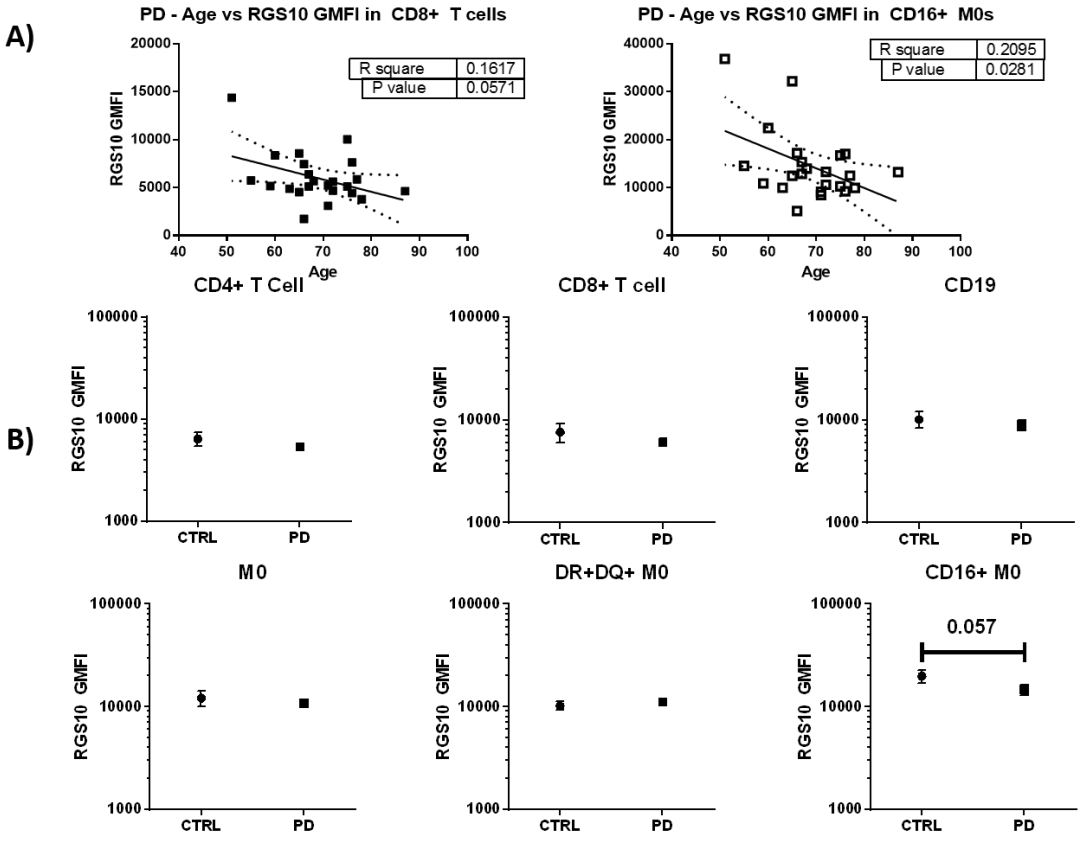

Figure 14. Decreased RGS10 expression is associated with age in CD8+ T cells and CD16+ monocytes in PD patients and decreased RGS10 expression in CD16+ monocytes relative to healthy controls. PBMCs from PD patients (n=22) and age-matched healthy controls (n=9) were analyzed for RGS10 expression by flow cytometry. We assessed the Pearson correlation between age and RGS10 expression in various immune cell subsets and was significant in CD8+ T cells and CD16+ monocytes from PD patients (A). Furthermore, the relative expression of RGS10 in immune cell subsets from healthy controls versus PD patients was determined by flow cytometry (B).

Chapter 4: Common genetic variant association with altered HLA expression, synergy with pyrethroid exposure, and risk for Parkinson's disease: An observational and case-control study
Previously published work[203].

4.1) Introduction

The etiology of Parkinson's disease (PD) remains largely unknown with less than 10% of cases attributable to an identifiable causative genetic mutation[54]. The clinical diagnosis of PD by its hallmark motor symptoms may be preceded by various non-motor symptoms, including depression, anosmia, constipation, and REM-sleep behavior abnormalities some of which have been postulated to be fueled by inflammatory processes[48,199]

Genetic polymorphisms in genes encoding glucocerebrosidase, α-synuclein, tau, and others have been reported to modify PD risk[54]. Environmental exposures, such as pesticide exposure and head trauma are associated with increased risk for developing PD[58,204]. Like other age-related diseases, current hypotheses suggest that genetic susceptibility must synergize with lifetime environmental exposures to initiate the development of PD pathology[205,206]. The major histocompatibility complex class II (MHC-II) that is responsible for antigen presentation to the adaptive immune system may be particularly important in linking genetic background to environmental exposures[113]. Inflammation has been implicated as a key driver of PD pathogenesis[77]. Post-mortem examination of PD brains has revealed microglial activation and lymphocyte infiltration in areas of degeneration[100,133]. Increased expression of inflammatory cytokines, altered composition of peripheral immune cells, and the protective effects of chronic ibuprofen consumption further implicate inflammation in PD pathogenesis[133,200,207].

The MHC-II locus contains the most highly polymorphic genes in the human population and mediates antigen presentation to CD4+ T cells and induction of adaptive immunity[113,114]. MHC-II molecules present antigenic peptides on the surface of antigen-presenting cells (APCs), such as B cells, monocytes, macrophages, dendritic cells, and microglia[113,114]. The MHC-II locus encodes three different α/β heterodimeric isotypes (HLA-DR, -DQ, and -DP)[113]. Each isotype has the potential to present distinct antigenic subsets to CD4+ T cells and induce their differentiation in a specified manner[113]. Differentiated CD4+ T cells (Th1, Th2, Th17, etc.) promote specific inflammatory effector responses or as regulatory T cells (Tregs), suppress inflammation[114]. Given its key role in adaptive immunity, the MHC-II locus is an ideal candidate for linking the environment and genetic susceptibility to PD pathogenesis through inflammation.

Supporting a disease-promoting role for antigen presentation, multiple studies have identified associations between single nucleotide polymorphisms (SNPs) in the MHC-II region and risk for late-onset PD[159-165,208-210]. In several genome-wide association studies (GWAS), the *rs3192882* SNP has been associated with altered risk for PD[159,160,211,212]; yet ethnic background appears to influence the allele associated with increased risk. In the largest GWAS to look at this SNP, homozygous carriers of the high-risk *G* allele (21% of PD patients and 16% of CTRLs) were found to have a 1.7 fold increased relative risk of developing PD in people of European ancestry[159]. Additionally, the *G* allele carried by 46% of PD patients and 40% of CTRLs was associated with increased levels of MHC-II as an expression-quantitative trait locus (eQTL) in subjects of European ancestry[208] and more strongly associated with risk for sporadic PD rather than familial PD[162]. As an eQTL, this SNP could be associated with genetic or epigenetic

regulatory elements that modify the expression of the MHC-II locus. ***These data led us to hypothesize that the rs3129882 GG genotype is associated with increased surface and mRNA expression and greater inducibility of the MHC-II locus in peripheral immune cells relative to the AA genotype.***

Given that the *rs3129882* SNP is located in the first intron of the monomorphic *HLA-DRA* gene and has not been associated with particular MHC-II haplotypes,[208] it was somewhat surprising that a common genetic variant in an immune locus could influence the risk for a complex neurologic disorder. Clearly, the genetic association between the MHC-II locus and risk for PD is complex and may depend on a variety of factors such as ethnic background, environmental exposures, etc. As such, ***we hypothesized that this SNP would synergize with pesticide exposure, a known PD-relevant risk factor, to increase risk for PD and this risk might be further modifiable by ethnicity and race.*** Given the heterogeneity of findings in various GWAS for MHC-II and risk for PD, the *rs3129882* SNP warranted further exploration as a possible genetic marker in certain populations associated with complex genetic and/or epigenetic mechanisms that modulate risk for PD by affecting antigen presentation.

4.2) Materials and Methods

4.2a) MHC-II Expression Cohort Subject Recruitment

PD patients and age-matched healthy CTRL subjects were recruited through the Clinical Research in Neurology (CRIN) IRB-approved research protocol at the Emory Movement Disorders Clinic and community outreach events sponsored by the American Parkinson's Disease Association, Wilkins Parkinson's Foundation, and Emory Udall

Center of Excellence for Parkinson's Reasearch. Participants were excluded if they were younger than 50 years old, were older than 85 years old, or had neurologic, chronic infectious, or autoimmune comorbidities, and/or known familial PD mutations. For subjects not originally in the Emory cohort of the Hamza et al., 2010 study in which participants' first HLA-DRA intron was sequenced, the *rs3129882* Taqman SNP Genotyping Assay (Life Technologies) was used to genotype newly recruited subjects. Subjects homozygous at the *rs3129882* locus were asked to provide a blood sample (~50 mL). At the time of recruitment, a questionnaire was used to assess disease and inflammation/immune-relevant environmental exposures and comorbidities. Caffeine, NSAID, and nicotine intake was calculated as mg-years, dose-years, and mg-years, respectively. Levodopa equivalence dose was calculated based on parameters defined by the Parkinson's Disease Society of the United Kingdom.

4.2b) Peripheral Blood Mononuclear Cell (PBMC) Isolation, Sorting, and Stimulation

PBMCs were isolated from whole blood using Ficoll-Paque (GE Healthcare) density centrifugation. The upper plasma layer was frozen immediately at -80°C. B cells and monocytes were isolated by positive selection from total PBMCs using anti-CD19 and CD14 paramagnetic beads, respectively (Miltenyi Biotec). Remaining cell fraction was analyzed by flow cytometry for quality control of sorting. For stimulation, monocytes were plated overnight with or without IFN-γ (PeproTech) in a 6-well plate at $5x10^5$ cells per well for flow cytometry or at $2x10^6$ cells per well for RNA isolation.

4.2c) RNA Isolation, cDNA synthesis, and RT-PCR

Cells were washed once in ice-cold phosphate-buffered saline and then lysed in 350 µL RLT buffer (Qiagen) supplemented with 1% β-mercaptoethanol (Sigma-Aldrich). Cell lysate was centrifuged through a Qiashredder (Qiagen) and then immediately frozen at -80°C. Later, RNA was fully isolated using RNAeasy Isolation Kit (Qiagen) and stored at -80°C. For cDNA synthesis, 0.5-2 µg of total mRNA was used per reaction in reverse transcription reactions using Superscript II (Life Technologies Corp) with oligo dT and random hexamer primers (Life Technologies Corp). The amount of SYBR-incorporated amplicons were measured for all real-time quantitative Bio-Rad iCycler instruments (Bio-Rad Laboratories) with an iQ optical module were used to measure the amount of SYBR incorporated amplicons for all real-time quantitative PCR reactions. DNA oligonucleotides (Integrated DNA Technologies) used for primers listed in **Table 2** were diluted to a final concentration of 100 nM for PCR reactions. All primers were tested by agarose gel electrophoresis to ensure that they formed single amplicon products of the correct size and optimized for Tm by temperature gradient real-time PCR followed by a melt curve analysis.

4.2d) Flow Cytometry Analysis

To stain for flow cytometry, $5x10^5$ cells per well were incubated in 1x FACS buffer (1% bovine serum albumin, 0.1% sodium azide, 1 mM EDTA) for 20 minutes at 4°C with anti-human HLA-DR:allophycocyanin/APC (1:20, BD Biosciences 559866), anti-human HLA-DQ FITC (1:20, BD Biosciences 347453), anti-human CD14:phycoerythrin/PE (1:100, Biolegend #301806) and anti-human CD19:peridinin chlorophyll/PerCP (1:100, Biolegend #302228). Cells were fixed with 1%

paraformaldehyde (Electron Microscopy Services) for 30 minutes at 4°C. Cells were stored in 1x FACS Buffer at 4°C until run on FACS Calibur within 1 week of staining. Spherobeads (BD Biosciences) and OneComp Beads (ebiosciences) were used to set voltages and compensation settings between runs. Analysis of flow cytometry data was performed on FlowJo Software v10.0.6.

4.2e) Mesoscale Discovery Multiplex ELISA

Plasma was obtained from the upper layer of Ficoll-Paque separation and stored at -80°C until sample analysis was performed. Plasma analyte levels were measured in duplicate using the Human Pro-inflammmatory Cytokine 7-plex, Human Chemokine V-PLEX, and Human CRP plates (Meso Scale Discovery). For measurement of CRP, samples were diluted 1:200. For all other assays, samples were measured undiluted.

4.2f) Genevar Analysis

Genevar 3.3.0 software (Wellcome Trust Sanger Institute) was used to interrogate the HapMap3 cis-eQTL database. Association of levels of cis-eQTLs within 500 kilobasepairs of the *rs3129882* SNP with SNP genotype for all the ethnic groups included in the database was reported as Spearman's rank correlation (rho) with p-value.

4.2g) Pesticide Exposure Cohort and Epidemiological Methods

The PEG case-control study recruited incident PD cases and controls from three highly agricultural counties in Central California, Kern, Fresno, and Tulare, between January 2001 and December 2013. Population-based controls were recruited from the

same tri-county study area and in the same age range as the cases using residential tax assessor's records. All subjects were required to have lived in California for at least 5 years and PD patients were examined at least once by our movement disorder specialists, multiple times, and met published criteria for idiopathic PD[213]. We described the details of case definitions[214] and subject recruitment[215] elsewhere. All procedures were approved by the University of California at Los Angeles (UCLA) Human Subjects Committee and informed consent was obtained from all participants**.**

In telephone interviews with patients and controls, we obtained detailed information on demographic characteristics, risk factors, and lifetime occupational and residential histories. We estimated ambient pesticide exposures resulting primarily from commercial applications to agricultural crops using a geographic information system (GIS) based computer model that links geocoded lifetime residential and occupations addresses of each participant to information on all commercial pesticide applications (date, location, and amount applied) from California State mandated pesticide use reports (CA-PUR) and land use data as published previously[216]. For each pesticide, we summed the pounds applied per year per acre within a 500-m radius buffer of an address since 1974 (year of CA-PUR mandate) and calculated study period average exposures for each subject and pesticide by summing the year-specific average exposures from 1974 to 10 years prior to the subject's index year (date of diagnosis for patients and interview for controls), and divided the sum by the number of years in the relevant time period. We substituted years missing a geocoded location with the average value of all recorded years for each pesticide and person. We then dichotomized exposures based on the

pesticide-specific median level in exposed controls (at or above). Participants could have received exposure at either residential or workplace addresses, or both.

The pesticide classes we examined were all previously identified as immunomodulatory[217]. Aside from paraquat, each of the pesticides fell into three different chemical classes: organophosphates, dithiocarbamates, and pyrethroids. After assessing exposure to individual pesticides, we counted the total number of pesticides in each group that each participant was exposed to at each location (residence/workplace address), and classified each participant as highly exposed (at or above the median number of pesticides in exposed controls) or receiving low/no exposure (none or below the median number of pesticides) based on the distribution for the total number of pesticides in exposed controls, except for the pyrethroids, where 75% of the exposed control population was only exposed to one pyrethroid pesticide, and we thus used the categories 'no' exposure versus exposure to one or more pyrethroid.

We used student's two-tailed t-test or a chi-square test to investigate differences in demographic factors between patients and controls. We assessed Hardy-Weinberg equilibrium for HLA *rs3129882* in control participants using a chi-square test and then evaluated association between the SNP and PD using logistic regression to calculate odds ratios (OR) and 95% confidence intervals (CI), assuming a recessive and additive genetic model as done in prior reports[159,212], and adjusted for potential confounders including age (continuous, at diagnosis for patients and interview for controls), sex, ever/never smoking status (having smoked at least 1 cigarette per week for at least a year). To assess gene-environment interactions between HLA *rs3129882* and pesticide groups, we introduced a multiplicative interaction term into a logistic model assuming an additive genetic model

and comparing only the *AA* and *GG* genotypes. Thus, we performed targeted analyses for specific chemicals (three different chemical classes and paraquat) selected *a priori* based on literature that suggest possible immune-modulatory effects of pesticides implicated in PD pathogenesis and for only one SNP. We used Quanto version 1.2.4[218] for power calculations and SAS 9.3 (SAS Institute Inc., Cary, NC) for all other analysis. To determine the *rs3129882* genotype of the PEG cohort, samples were coded to blind the researchers and the *rs3129882* Taqman SNP Genotyping Assay (Life Technologies) was used for genotyping.

4.2h) Statistical Analyses

A one-tailed Students' t-test was used to make comparisons between the high risk and low risk genotype groups in the immunophenotyping studies at Emory University. One-way and two-way analysis of variance followed by Holm-Sidak post-hoc tests were used when to compare the baseline characteristics of the study population and inducibility of the response to IFNγ between the high risk and low risk, respectively, as indicated in figure legends. The Pearson coefficient was used to assess the correlation between two variables. Graphpad (Prism) and R (GNU project) software was used to perform statistical analyses. See Pesticide Exposure Cohort and Epidemiological Methods section above for detailed description of statistical methods used to interrogate the pesticide exposure cohort.

4.2i) Study approval

Written informed consent was received from participants prior to inclusion in the study. All participants provided written informed consent prior to inclusion in the research studies, and all procedures were approved *a priori* by the Institutional Review Board of Emory University in Atlanta, Georgia or of UCLA in Los Angeles, California.

4.3) Results

4.3a) MHC-II Expression Study Population

To assess the effect of *rs3129882* on MHC-II expression, 81 homozygous non-PD control (CTRL) and PD subjects were recruited into four groups: CTRL *AA* (n=25), CTRL *GG* (n=12), PD *AA* (n=15), and PD *GG* (n=29). This strategy allowed us to examine the effect of SNP genotype, as well as disease status, on MHC-II expression. The groups of this study population were balanced with respect to factors that modify PD risk[59,60,69], including age, smoking, non-steroidal anti-inflammatory drug (NSAID) use, caffeine intake, mean Unified Parkinson's Disease Rating Scale (UPDRS) motor score, levodopa equivalence dose, and duration of disease for the PD groups (**Table 3**).

4.3b) The rs3129882 GG genotype is associated with increased surface MHC-II expression

Given that the *rs3129882 G* SNP was associated with increased levels of MHC-II eQTL in subjects of European ancestry[208], we tested the hypothesis that in people of European ancestry, homozygosity for the *G* SNP would be associated with higher MHC-II expression compared to homozygosity for the *A* SNP. Flow cytometry was used to measure the frequency of cells expressing both HLA-DR and –DQ (DR/DQ double-

positive cells) on the cell surface of APCs. We focused our analyses on B cells and monocytes because they are the major APCs in the peripheral blood. Nearly all peripheral B cells and monocytes were HLA-DR positive (**Fig 15**). In our cohort, 80% of peripheral blood B cells were HLA-DR/DQ double-positive in all four groups of subjects (**Fig 16A**). Similarly, nearly all monocytes were HLA-DR positive but only 20-30% of peripheral blood monocytes were HLA-DR/DQ double-positive in all four groups of subjects (**Fig 16A**). Using flow cytometry, the average level of HLA-DR or HLA-DQ surface expression was also measured, reported as median fluorescence intensity (MFI). In both B cells and monocytes from the CTRL *GG* group, there was a significant two-fold increase in the MFI of HLA-DR compared to the CTRL *AA* group (**Fig 16B**). The MFI of HLA-DQ on B cells from *GG* individuals was also significantly increased in both CTRL and PD patients by 1.5- to 2-fold (**Fig 16C**). In this cohort, having the *GG* genotype was associated with increased MFI of HLA-DR on both B cells and monocytes and of HLA-DQ on B cells.

4.3c) The rs3129882 GG genotype is associated with increased IFNγ inducibility of HLA-DQ expression

Given that MHC-II expression increases upon cellular activation[113,219], we also measured disease- and genotype-specific effects on induction of this locus using IFN-γ, a potent stimulator of MHC-II gene expression[219,220]. In response to IFN-γ, the frequency of monocytes that became HLA-DR/DQ double-positive cells was significantly higher in the PD *GG* group relative to the PD *AA* group (**Fig 16D**). The level of induction of HLA-DR surface expression on monocytes was the same in all four groups of subjects (**Fig**

16E). In contrast, the HLA-DQ surface expression was significantly increased on IFNγ stimulated monocytes from CTRL *GG* individuals compared to CTRL *AA* individuals (**Fig 16F**). In PD *GG* patients, HLA-DQ surface expression was significantly increased relative to PD *AA* patients at the highest dose of IFNγ (**Fig 16F** inset). In summary, the *rs3129882 GG* genotype was associated with increased HLA-DQ expression on monocytes in response to IFN-γ.

4.3d) The rs3129882 GG genotype is associated with increased baseline expression and IFNγ inducibility of MHC-II mRNA

Next, we interrogated transcriptional levels of MHC-II to determine possible mechanisms underlying the association of *rs3129882* with altered surface MHC-II expression. To assess the extent to which APCs from *AA* versus *GG* individuals expressed differences in mRNA levels of the various MHC-II isotypes, we performed quantitative RT-PCR for the four MHC-II genes in closest proximity to *rs3129882*: *HLA-DRA*, *HLA-DRB1*, *HLA-DQA1*, and *HLA-DQB1* that are common among all MHC-II haplotypes. In B cells from CTRL subjects, the high-risk genotype was significantly associated with increased mRNA levels of *HLA-DRA, -DRB1,* and *-DQB1* (**Fig 17A**). In monocytes, the *GG* genotype was associated with increased *HLA-DQB1* mRNA expression in both PD patients and CTRLs and with increased *HLA-DRB1* mRNA expression in PD subjects (**Fig 17B**). In addition to these statistically significant 10- to 20-fold increases in mRNA expression, there were clear upward trends in expression of *HLA-DRB1* and *-DQB1* mRNA in B cells from PD *GG* patients and *HLA-DRB1* mRNA expression in monocytes from CTRL *GG* subjects.

Following IFNγ stimulation, monocytes from PD *GG* patients displayed a >200-fold increase in mRNA levels of all measured MHC-II genes relative to PD *AA* patients and CTRLs of either genotype (**Fig 17C**). With the exception of significantly decreased inducibility of *HLA-DQA* mRNA in the CTRL *GG* group, the inducibility of the rest of the MHC-II genes was the same in PD *AA* patients and CTRLs of either genotype. In summary, the *GG* genotype was associated with significantly increased *HLA-DRA*, *-DRB1,* and *-DQB1* mRNA expression in resting B cells independent of disease. In resting monocytes, the high-risk genotype was also associated with increased *HLA-DRB1* and *HLA-DQB1* mRNA expression independent of disease. Finally, monocytes treated with IFN-γ from the PD *GG* group displayed higher levels of all MHC-II mRNA.

4.3e) The rs3129882 high-risk genotype is associated with increased plasma CCL-3 (MIP-1α) levels in PD patients but not with altered frequencies of B cells and monocytes in the peripheral blood

In conjunction with cell-specific markers, differences in peripheral blood mononuclear cell (PBMC) composition and blood levels of cytokines/chemokines can indicate an active inflammatory process. Flow cytometry analysis of Ficoll-Paque separated PBMCs demonstrated no change in B cell or monocyte frequency between any of the study groups (**Fig 18A**). The levels of 17 selected immunomodulatory cytokines and chemokines were measured by multiplexed chemiluminescent immunoassays (**Fig 18B**). Individuals in the PD *GG* group displayed increased circulating plasma levels of CCL-3 (also known as MIP-1α) greater than two-fold over the PD *AA* group. The levels

of the other 16 cytokines and chemokines were not significantly different between the four groups (**Fig 18B**).

4.3f) Pyrethroid exposure and the high-risk rs3129882 genotype increases odds for PD

To determine whether there are interactions between pesticide exposure and *rs3129882* in PD, 465 incident PD patients (diagnosed within 3 years of recruitment) and 497 population controls of European ancestry from the Parkinson's Environment and Gene (PEG) case-control study were examined. Basic demographic characteristics for the PEG study population can be found in **Table 4**. As expected, patients were more likely to be male and have a family history of PD, and less likely to have ever been smokers[48,49,59]. Our control population was in Hardy-Weinberg equilibrium for HLA *rs3129882* (p= 0.18). Genotype alone did not significantly influence PD risk, assuming previously reported genetic models (additive model: OR=1.03, 95% CI=0.86, 1.24 for those with one risk allele (*AG*) and OR=1.07, 95% CI=0.74, 1.55 for those with two risk alleles (*GG*); recessive model: OR=0.83, 95% CI=0.59, 1.16; **Table 5**)[159,212]. We did not detect interactions with organophosphates, dithiocarbamates, or paraquat and *rs3129882*. Investigating pyrethroid pesticides, in logistic regression models we estimated a positive interaction on a multiplicative scale when comparing groups homozygous at the SNP (*AA* versus *GG*; interaction p-value=0.02; **Table 6**), and when we used an additive genetic model to include heterozygous individuals (interaction p-value=0.007; **Table 6**). In both models, neither the genotype alone nor pyrethroid exposure alone significantly influenced PD risk, but in those jointly exposed to pyrethroids and having a *GG* genotype we estimated significant increases in PD risk. For example, when comparing *AA* versus *GG*

in those unexposed, we see a slight non-significant decrease in PD risk (OR=0.73, 95% CI=0.47, 1.14), and no effect when comparing pyrethroid exposure in those with the *AA* genotype (OR=1.04, 95% CI=0.65, 1.67), yet comparing those homozygous for the risk allele (*GG*) and exposed to pyrethroids to those with the *AA* genotype and unexposed, we see an increased risk of PD (OR=2.48, 95% CI=1.24, 4.97; **Table 6**).

Next, we assessed whether there were differences in disease characteristics of the *AA* vs *GG* individuals at baseline and during two follow up exams in a subset of the PEG population followed over time; mean years between baseline and exam 1 was 3.5 years (SEM=0.1) and exam 2 was 5.6 years (SEM=0.2). We did not see any significant differences in measures of disease-related clinical progression between *AA* and *GG* individuals at baseline or either follow-up time point (**Table 7**).

4.3g) Genetic variation associated with ethnicity can reverse allelic rs3129882 association of MHC-II expression changes

To contextualize the discrepancies among various GWAS with associations of the *rs3129882* with risk for PD, we used the GeneVar software tool to interrogate the HapMap3 cis-eQTL database. The HapMap3 database is unique in that it consists of 726 lymphoblastoid cell lines developed from individuals of 8 different ethnic groups. We tested the hypothesis that association of SNP genotype with cis-eQTL level in the MHC-II locus would be affected by ethnicity. Indeed, we observed that in the individuals of the HapMap3 database, the allele that was associated with increased eQTL level depended on ethnicity (**Table 8**). For example, the *G* allele was associated with increased levels of *HLA-DRB1* eQTL in Utah Caucasians (rho=0.199, p=0.038) but with decreased levels in

Han Chinese (rho=-0.281, p=0.0124) and Nigerian Yoruba (rho=-0.317, p=8.0E-4). Furthermore, *HLA-DRB5* eQTL level was positively associated with the *G* allele in Utah Caucasians (rho=0.516, p=9.3E-9) but negatively associated with the *G* allele in most of the other ethnic groups.

4.4) Discussion

The MHC-II locus and particularly the *rs3129882* SNP, has been implicated in modulating risk for PD[159,160,164]; and herein, we demonstrate that the *G* allele of this SNP, acting together with environmental pyrethroids exposure, increases the odds of developing PD and is associated with altered MHC-II expression in peripheral APCs. The association of the high risk genotype with altered MHC-II expression was revealed by: 1) increased surface protein expression of HLA-DR in monocytes; 2) greater inducibility of HLA-DQ surface protein expression in monocytes in response to IFN-γ; 3) increased mRNA expression of *HLA-DRA, -DRB1,* and *-DQB1* genes; and 4) greater inducibility of mRNA expression in PD patients' monocytes after IFNγ stimulation. Notably, within our MHC-II expression study cohort more PD patients tended to be male and controls, who were often caregivers, tended to be women. Despite this unequal sex distribution, stratification of the data by sex did not account for statistical differences in MHC-II expression (data not shown). Taken together, the data indicate that in our cohort, *GG* homozygosity at the *rs3129882* SNP is associated with increased baseline and inducible MHC-II expression in APCs. This immune hyper-responsiveness may in turn be one reason why this SNP has been associated with altering risk for late-onset PD.

Specifically, the findings revealed that monocytes from individuals in our cohort with the *G* allele have more antigen-presenting capacity under resting conditions; and that

within the context of PD, cytokine challenge increases mRNA expression of MHC-II genes approximately 200-fold. Surprisingly, plasma levels of cytokines were unchanged among all four groups except for increased MIP-1α in the PD GG group relative to the PD AA group, suggesting that immune risk for PD may be better predicted by cell-associated immune molecules rather than measurement of global fluid biomarkers. The pathologic significance of an isolated elevation in a single plasma chemokine is unclear. These novel findings directly implicate regulation of MHC-II expression and antigen presentation as important mechanisms underlying the reported association between late-onset PD and the MHC-II locus. This evidence supports a role for immunological processes and the synergy between these processes and environmental exposures such as pyrethroids in determining an individual's susceptibility to PD.

On the basis of the studies reported here, we speculate that the *rs3129882* SNP is a marker in some populations for a genetic and/or epigenetic mechanism(s) that control the expression of the MHC-II locus. A common mechanism in the MHC-II locus that promotes risk for PD may exist in many different human populations but may be marked by different disease-associated SNPs. Evidence for this is indicated by the divergent associations reported in GWAS in Han Chinese cohorts where the *A* allele is associated with increased PD risk while in European cohorts, the *G* allele is associated with increased risk[159,160,211,212]. The ethnicity-dependent directional changes in the HapMap3 database cis-eQTL associations in **Table 8** also point to the importance of taking into account ethnic differences when studying genetic variation at the MHC-II locus. Thus, the association of various MHC-II SNPs with PD likely depends heavily on ethnic make-up and exposome.

The expression of genes at this locus is regulated by a complex interplay between transcription factors, chromatin architecture, DNA methylation sites, and histone remodeling [221]. In unstimulated cells from *GG* individuals in our cohort, mRNA expression of *HLA-DRA*, *DRB1*, and *DQB1* is increased, suggesting a mechanism that allows for basal increases in transcription of these genes (**Fig 17A, 17B**). The increase in the levels of β-subunit mRNAs is particularly interesting because the α-subunit mRNAs are typically much more abundant, making the level of β-subunit mRNAs an important limiting factor in production of mature MHC-II molecules. However, only in PD patients was the *rs3129882 G* allele associated with increased IFN-γ inducibility of mRNA expression of all the MHC-II genes. In CTRLs, the IFN-γ inducibility of *HLA-DQA* and *HLA-DRA* mRNA expression was significantly decreased (**Fig 17C**). This phenomenon suggests that in controls, a regulatory mechanism may exist to limit the level of mRNA expression of MHC-II, i.e. a "ceiling effect" upon APC activation. If this turns out to be the case, our data suggest this regulatory mechanism may be absent (or perhaps is lost as a result of the disease process itself) in individuals with a SNP in the MHC-II locus that hence increases risk of PD. Irrespective of the mechanism, enhanced transcription of MHC-II genes would be expected to increase surface expression of these molecules, resulting in functional consequences for engagement of the adaptive immune system.

Engagement of the adaptive immune system through MHC-II peptide presentation would allow for a specific, chronic inflammatory response mediated by CD4+ T cells[114]. Shifts in the overall levels of MHC-II and/or the relative levels of MHC-II isotypes could impact normal immunologic processes in two main ways. First, antigenic peptide epitopes that promote pathogenesis leading to PD may be more likely to be presented to

the adaptive immune system and dominate immune responses[222]. This could occur through skewed expression of HLA-DQ proteins. Second, the presentation of certain peptides on different MHC-II isotypes could alter the differentiation of CD4+ T cells into various subsets (i.e., Th1, Th2, Th17, or Tregs)[223]. Evidence for such phenomena can be demonstrated in human autoimmune disease and humanized rodent models of autoimmunity[224]. In this manner, antigen presentation via MHC-II on the surface of APCs to CD4+ T cells is a mechanism that could link the contribution of both genetic background and environmental exposure to susceptibility for developing PD.

In the context of risk for PD, two people who are exposed to the same insult are likely to respond very differently immunologically given their genetic and epigenetic background.

Within the context of our findings, following exposure to an environmental agent such as pyrethroids or an event such as traumatic brain injury, we would predict that an individual with the *AA* genotype in our cohort (**Fig 19A**) might display an immune response that resolves completely in a few weeks. By contrast, an individual from our cohort with the *GG* genotype (**Fig 19B**) might display a heightened immune response that may not resolve quickly but may instead promote chronic neuroinflammation, thereby hastening degeneration of vulnerable neuronal populations. Indeed, an adaptive immune response that is propagated in response to highly specific antigens differentially presented by APCs from people with high-risk versus low-risk MHC-II alleles can explain the selective degeneration of vulnerable neuronal populations as is the case in PD.

In agreement with our understanding of risk for PD, environmental stimuli, such as pyrethroids, are likely to play a prominent role in synergizing with the differential immune responses associated with this SNP given that few HLA haplotype associations

have been reported for PD risk and none segregate with this SNP[208]. Pyrethroid exposure has been associated in humans with acute alterations in immunoglobulin levels and T cell frequencies[225] and in animal models with altered APC function[226]. Related to Parkinson's disease, pyrethroids are associated with increased striatal dopamine uptake, increased striatal dopamine metabolism, and altered electroencephalographic activity in the SN in chronically exposed rodent models[227-229]. Other pesticides have been shown to directly impact antigen presentation and although this has not been reported for pyrethroids it is possible their effects on immune cells may explain the synergism with the MHC-II locus to increase risk for PD[230]. The identified combined risk conferred by the *rs3129882 G* allele and pyrethroids may be explained by complex interactions that impact both the nervous system and the immune system to initiate PD pathogenesis through voltage-gated sodium channels that are known targets of this class of pesticides. The follow-up of the PEG patients only cohort suggested no association between the *G* allele and disease severity/progression (**Table 7**). However, longer follow-up time may be necessary to reveal differences in disease progression that are not yet apparent in this cohort. It is important to note that subject recruitment occurred through a state-wide registry with 30 years of exposure data; and PD patients underwent a neurologic examination by movement disorder specialists. Our study is the first population-based analysis to explore and report pyrethroid-gene variant interactions; and thus, it requires replication in an independent sample. Longitudinal studies of the MHC-II locus and PD would clarify the role of this locus in increasing risk.

The significance of our findings is three-fold. First, we provided direct evidence from human peripheral blood that implicates a mechanism for antigen presentation and

the role of adaptive immunity in risk for PD. Functional studies that link GWAS hits to functional cellular changes are the next step in understanding how genetics, environment, and cellular responses synergize to increase risk for complex diseases. Second, our findings suggest that the level and quality of MHC-II expression could prove to be an effective immune marker for the prediction of disease susceptibility in addition to honing our understanding of disease pathogenesis. Third, our data suggest that cellular biomarkers may prove more useful than soluble molecules in plasma and CSF to identify individuals at risk for disease or for patient recruitment into neuroprotective trials testing immunomodulatory drugs.

4.5) Figures

Target	Forward Primer (5'-3')	Reverse Primer (5'-3')
HLA-DRA	**GAGTTTGATGCTCCAAGCCCTCTCGC**	**CAGAGGCCCCCTGCGTTCTGCTGCA**
HLA-DRB1	**TGCTGAGCTCCCTACTGGCT**	**CGCGTACTCCTCTCGGTTATAG**
HLA-DQA	**CACCTTTTCTCTGGGACTTAAGC**	**TGAGGAATTAGGTAGCCGGGT**
HLA-DQB	**TATGCCTGCCCAGAATTCCC**	**CCATCAAGGCGGACCATGTGT**
18S rRNA	**GTAACCCGTTGAACCCCATT**	**CCATCCAATCGGTAGTAGCG**

Table 2. RT-PCR Primers. List of primers used for qRT-PCR to measure mRNA expression of MHC-II genes.

	CTRL AA	CTRL GG	PD AA	PD GG	All	p-value
N	25	12	15	29	81	
Age	65.5 ± 1.8	63.7 ± 1.8	67.4 ± 2.2	68.5 ± 1.5	66.6 ± 0.9	0.38
Sex	19F, 6M	11F, 1M	6F, 9M	9F, 20M	45F, 36M	0.003
Smoking (pack-yrs)	2.9 ± 1.9	7.5 ± 3.2	8.3 ± 4.6	12.2 ± 5.0	7.8 ± 2.1	0.43
Caffeine (mg-yrs)	6856 ± 1822	7959 ± 2068	5913 ± 1385	5907 ± 1341	6515 ± 806	0.49
NSAID use (dose-yrs)	1.95 ± 0.71	3.37 ± 1.3	4.34 ± 1.2	3.72 ± 1.0	3.30 ± 0.52	0.36
Number of Lifetime Head Injuries	0.22 ± 0.15	0.78 ± 0.36	0.47 ± 0.22	0.67 ± 0.26	0.50 ± 0.12	0.49
Age at Diagnosis			58.5 ± 3.0	59.5 ± 1.6	59.2 ± 1.5	0.40
Years with Disease			8.5 ± 2.0	9.0 ± 1.1	8.8 ± 0.95	0.78
Mean UPDRS Score			15.4 ± 2.9	16.8 ± 1.5	16.4 ± 1.4	0.41
Levodopa Equivalence Dose (mg)			222.5 ± 37.2	261.2 ± 45.5	244.4 ± 30.11	0.53

Table 3. Characteristics of MHC-II Expression Study Population.

Data are expressed as the mean ± SEM for each of our four groups (CTRL AA, CTRL *GG*, PD *AA*, PD *GG*) and the entire study population. The table shows that our four groups are well balanced for the age, the self-reported environmental exposures, and clinician reported disease severity (UPDRS) measures. One-way ANOVA indicates no significance between groups at $p > 0.05$ for age, smoking, caffeine, NSAID use, number of lifetime head injuries, age at diagnosis, years with disease, mean UPDRS score, and

levodopa equivalence dose. Chi-square test indicates significant difference (p = 0.003) in proportion of males to females in groups but stratification of data by sex does not explain statistical differences in MHC-II expression (data not shown).

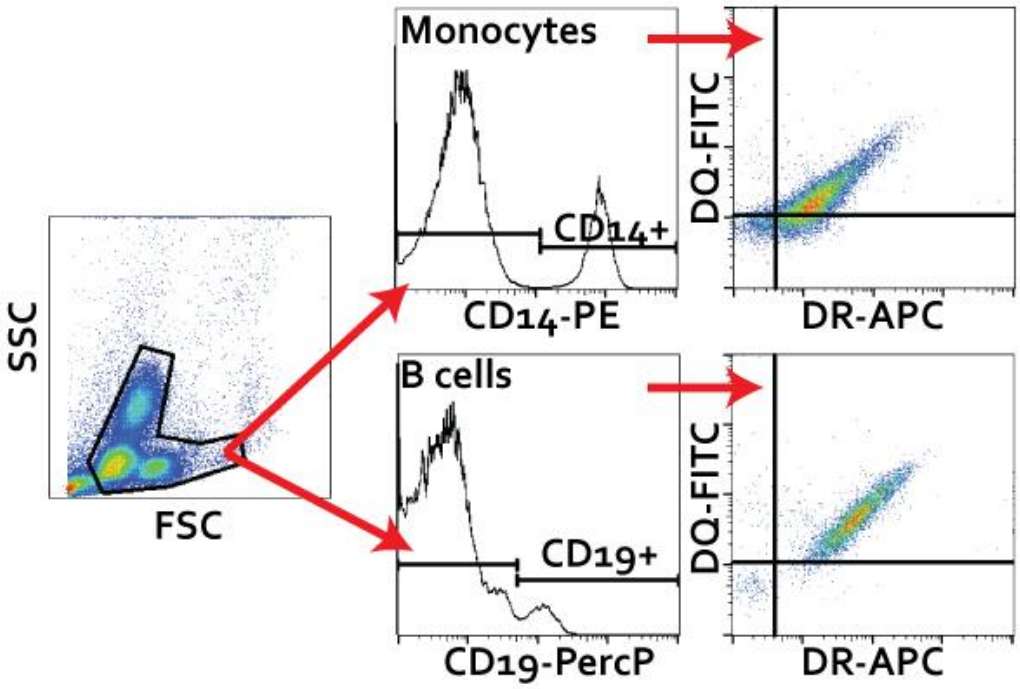

Figure 15. Gating Strategy for Flow Cytometry Analysis. Cells were stained for flow cytometry and analyzed on a FACS Calibur after standardization and compensation with Sphero beads, OneComp beads, and Raji and THP-1 cell lines. Gates were placed based on staining with isotype control antibodies. Analysis was performed and plots were generated using FlowJo software.

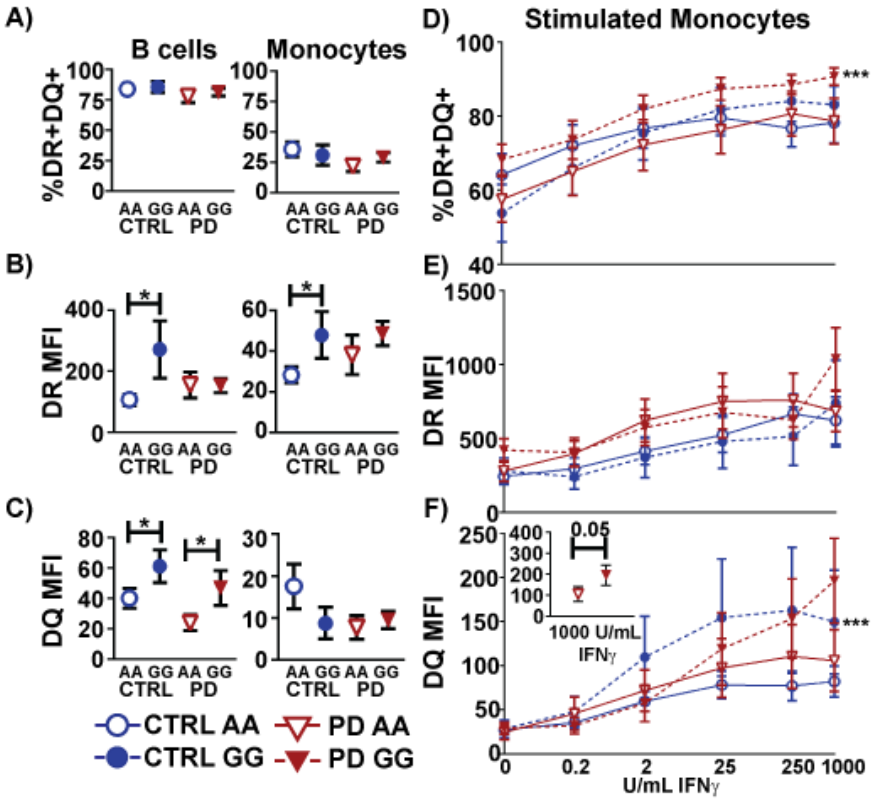

Fig 16. The high-risk *rs3129882* GG genotype is associated with an increased level of MHC-II expression in B cells and monocytes and with increased inducibility of surface HLA-DQ expression

A) Frequency of HLA-DR/DQ double-positive, B) average level of HLA-DR expression, C) average level of HLA-DQ expression in B cells and monocytes was determined by flow cytometry staining of total PBMCs. One-tailed student's T-test between high risk and low risk allele groups was used to test for significance. * $p < 0.05$. HLA-DR MFI: CTRL AA vs CTRL GG B cells $t(33) = 2.28$ $p < 0.05$; Monocytes $t(34) = 2.14$ $p < 0.05$. HLA-DQ MFI CTRL AA vs CTRL GG B cells $t(28) = 1.76$, $p < 0.05$; PD AA vs PD GG $t(35) = 1.82$, $p < 0.05$. Surface MHC-II expression in paramagnetically, positively-sorted monocytes stimulated with various concentrations of IFNγ was measured by flow cytometry staining to measure increase in D) frequency of HLA-DR/DQ double-positive

cells, E) level of HLA-DR expression, and F) level of HLA-DQ expression. Two-way ANOVA was performed to test for significance between GG and AA groups. *** $p < 0.001$. 2A) PD GG vs PD AA $F(1,190) = 11.97$ $p < 0.001$. 1F) CTRL GG vs CTRL AA $F(1, 163) = 10.39$ $p < 0.001$. Inset for DQ MFI (1F) panel shows sorted monocytes from PD patients stimulated with 1000 U/mL IFN-γ. One-tailed T-test was performed for significance. $t(31) = 1.52$; $p = 0.05$.

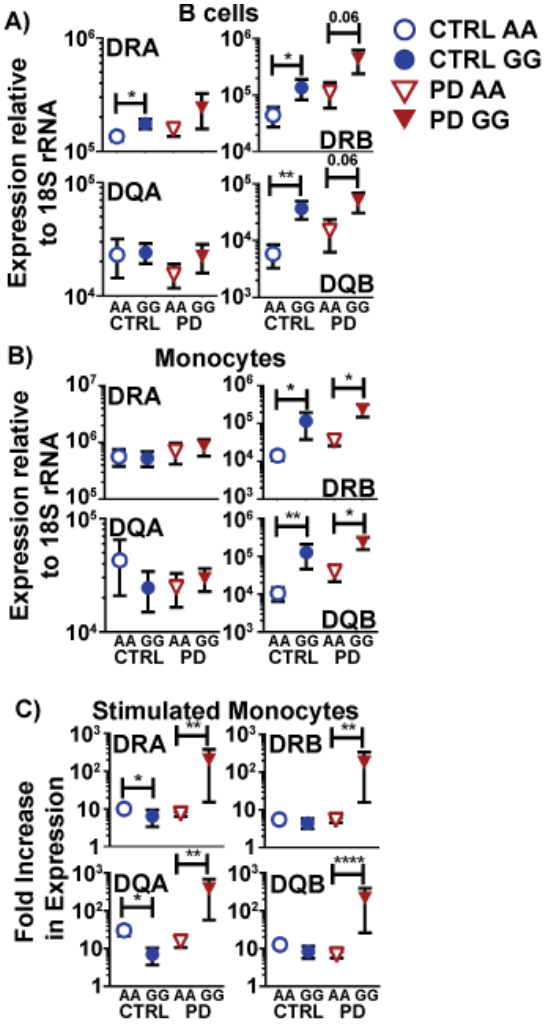

Fig 17. The high-risk *rs3129882* GG genotype is associated with increased baseline expression and inducibility of MHC-II mRNA

RNA was isolated from paramagnetically, positively sorted A) B cells and B) monocytes. MHC-II mRNA expression was quantified relative to 18s rRNA with RT-PCR.* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. B cell CTRL GG vs CTRL AA *HLA-DRA* $t(35) = 2.01$ $p < 0.05$; *HLA-DRB1* $t(34) = 2.04$ $p < 0.05$; *HLA-DQB1* $t(33) = 3.28$ $p < 0.01$. B cell PD *GG* vs PD *AA* *HLA-DRB1* $t(25) = 1.59$ $p = 0.06$; *HLA-DQB1* $t(33) = 1.64$ $p = 0.06$. Monocytes CTRL *GG* vs CTRL *AA* *HLA-DRB1* $t(32) = 1.90$ $p < 0.05$; *HLA-DQB1* $t(35) = 2.08$ $p < 0.01$; PD *GG* vs PD *AA* *HLA-DRB1* $t(26) = 2.24$ $p < 0.05$; *HLA-DQB1* $t(30) = 2.28$ $p < 0.05$ C) Fold change in MHC-II expression with or without 100 U/mL IFNγ stimulation in paramagnetically, positively sorted monocytes was measured by RT-PCR after normalization to 18s rRNA levels. One-tailed T-test was performed as indicated. **** $p < 0.0001$ ** $p < 0.01$, * $p < 0.05$. CTRL AA vs GG *HLA-DRA* $t(33) = 1.77$ $p < 0.05$; *HLA-DQA1* $t(13) = 1.80$ $p < 0.05$; PD AA vs PD GG *HLA-DRA* $t(36) = 1.76$ $p < 0.01$; *HLA-DRB1* $t(29) = 1.65$ $p < 0.01$; *HLA-DQA1* $t(24) = 1.82$ $p < 0.01$; *HLA-DQB1* $t(30) = 2.53$ $p < 0.0001$.

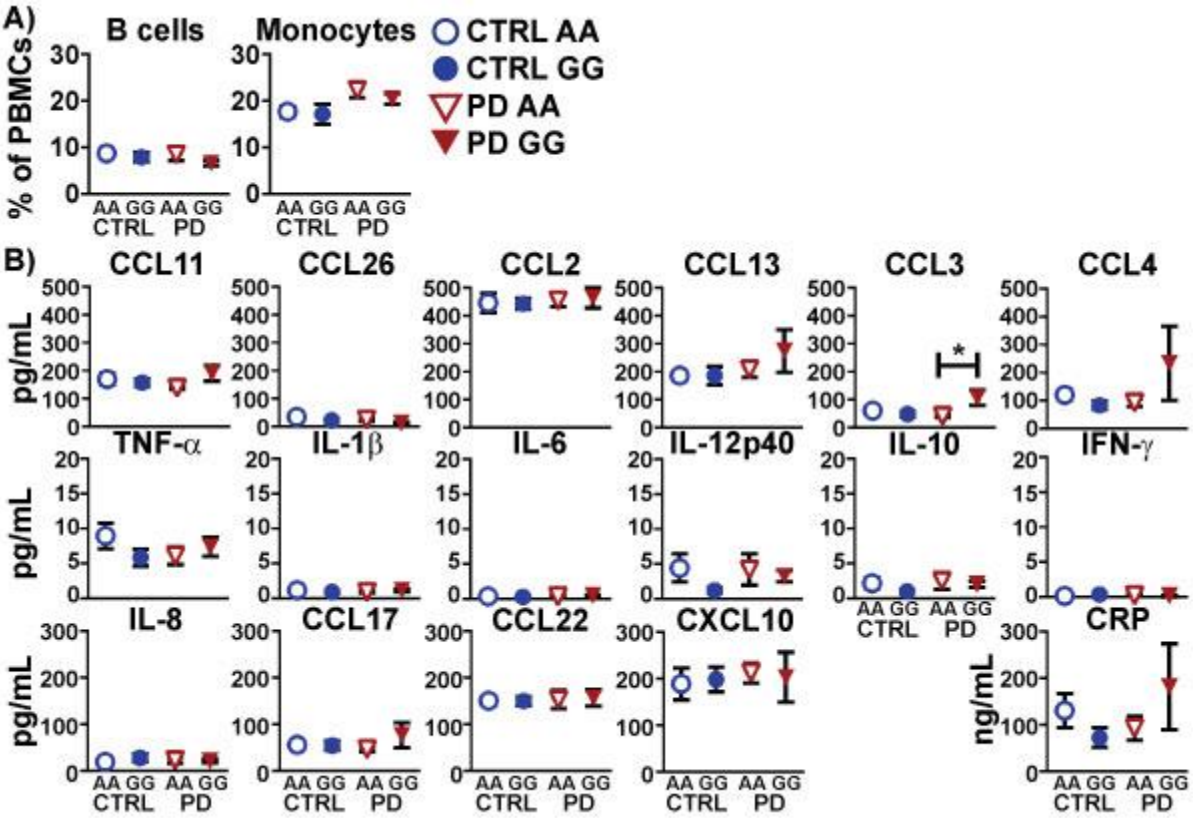

Fig 18. The *rs3129882* high-risk genotype is associated with increased plasma CCL-3 levels in PD patients with the high-risk *rs3129882* GG genotype but not with altered frequencies of B cells and monocytes in the peripheral blood.

A) B cell and monocyte frequencies were determined by flow cytometry staining of total PBMCs. B) Mesoscale Discovery (MSD) Multi-Array immunoassay technology was used to measure plasma levels of the indicated cytokines and chemokines. Values are plotted as the mean ± SEM. Two-tailed student's T-test was used to test for significance between high risk versus low risk allele. * $p < 0.05$. CCL-3 PD GG vs PD AA $t(16) = 2.39$, $p < 0.05$.

Characteristic	Patients (n=465)	Controls (n=497)	P-Value[a]
Age[b], mean ± SEM	69.4 ± 0.46	67.4 ± 0.52	0.005
Age at PD onset, mean ± SEM	67.8 ± 0.53		
Male sex, n (%)	289 (0.52)	262 (0.48)	0.003
Family history of PD[c], n (%)	76 (0.16)	47 (0.09)	0.001
Ever cigarette smoker, n (%)	212 (0.46)	271 (0.54)	0.005
Years with Disease, mean ± SEM	2.49 ± 0.10		
UPDRS-III Score[d], mean ± SEM	21.0 ± 0.48		

[a]P-values based on comparison between cases and controls using with chi-square or t-test
[b]Age at PD diagnosis for patients and interview for controls
[c]First degree relative with PD; 3 patients missing PD family history
[d]17 patients missing UPDRS-III score

Table 4. General characteristics of PEG study population of European ancestry, n=962 (patients=465, controls=497).

Data are expressed as the mean ± SEM for patients versus controls. The table shows that the PD patient group in the PEG study population is slightly older, more likely to have a family history of PD, has more males, and people with a history of cigarette smoking.

Genotype	Cases n (%)	Controls n (%)	Adjusted OR (95% CI)[a]	P-Value
AA	142 (0.31)	175 (0.35)	1.00 (ref)	--
AG	243 (0.52)	227 (0.46)	1.03 (0.86, 1.24)	0.72
GG	80 (0.17)	95 (0.19)	1.07 (0.74, 1.55)	
		AA vs GG:	0.95 (0.65, 1.38)	0.78
		AA/AG vs GG	0.83 (0.59, 1.16)	0.27

[a]Adjusted for age (continuous), sex, and smoking history.

Table 5. HLA-DRA *rs3129882* marginal effects in PEG population, n=962 (patients=465, controls=497).

An additive model was used to assess the association between the *rs3129882 G* allele and Parkinson's disease in the PEG population. Increased odds of developing PD was not associated with genotype alone in this population

Ambient Pyrethroids[b]	***None***				***1+ pesticide***			
	Cases n (%)	**Controls n (%)**	**Adjusted OR[a] (95% CI)**	**p value**	**Cases n (%)**	**Controls n (%)**	**Adjusted OR[a] (95% CI)**	**p value**
Additive Genetic Model								
AA	95 (0.30)	117 (0.33)	1.00 (ref)		47 (0.32)	58 (0.42)	0.83 (0.53, 1.28)	0.42
AG	172 (0.54)	161 (0.45)	0.91 (0.73, 1.13)	0.38	71 (0.48)	66 (0.48)	1.25 (0.88, 1.78)	0.22
GG	50 (0.16)	81 (0.23)	0.82 (0.53, 1.27)		30 (0.20)	14 (0.10)	1.87 (1.08, 3.35)	0.03
							P=value for interaction:	*0.02*
AA vs GG								
AA	95 (0.66)	117 (0.59)	1.00 (ref)		47 (0.61)	58 (0.81)	1.04 (0.65, 1.67)	0.87
GG	50 (0.34)	81 (0.41)	0.73 (0.47, 1.14)	0.17	30 (0.39)	14 (0.19)	2.48 (1.24, 4.97)	0.01
							P=value for interaction:	*0.007*

[a]Adjusted for age (continuous), sex, and smoking history.

[b]Ambient pesticide exposure to any pyrethroids (at or above the median level seen in exposed controls) at both occupation and residence, from 1974 (year of CA-PUR implementation) to 10 yrs before diagnosis or interview. Pyrethroid group includes fenvalerate, permethrin, phenothrin, resmethrin, flucythrinate, cypermethrin, (S)-cypermethrin, tau-fluvalinate, fenpropathrin, lamda-cyhalothrin, bifenthrin, esfenvalerate, and tralomethrin; Cyfluthrin had no exposure in study popluation.

Table 6. Interaction, main, and joint effect estimates between HLA *rs3129882* and pyrethroid exposure in PEG study population of European ancestry, using both an additive genetic model and AA vs GG; n=962 (patients=465, controls=497).

Using both an additive genetic model and comparing only the homozygous groups, we assessed the association between pyrethroid exposure and the risk *rs3129882* genotype in

the risk for PD. The table indicates the adjusted odds ratio and p-values for the main and joint effects and the p=value for interaction.

	Baseline with follow-up (n=88)		**Follow-up 1 (n=88)**		**Follow-up 2 (n=69)**	
	AA (n=53)	GG (n=35)	AA (n=53)	GG (n=35)	AA (n=41)	GG (n=28)
Age at PD diagnosis	65.3 ± 1.4	69.3 ± 1.6	--	--	--	--
Age at PD onset	63.7 ± 1.8	67.3 ± 2.1	--	--	--	--
PD Duration (years)	2.0 ± 0.2	1.6 ± 0.2	5.8 ± 0.4	5.1 ± 0.4	7.8 ± 0.5	7.2 ± 0.5
Follow-up (years)	--	--	3.8 ± 0.3	3.4 ± 0.3	5.8 ± 0.3	5.5 ± 0.4
Clinical features						
MMSE Score	28.6 ± 0.2	28.5 ± 0.3	28.3 ± 0.3	28.2 ± 0.4	28.0 ± 0.4	27.4 ± 0.6
GDS	3.1 ± 0.5	2.4 ± 0.4	3.7 ± 0.4	3.7 ± 0.6	3.3 ± 0.5	3.4 ± 0.5
UPDRS Score	17.7 ± 1.0	17.0 ± 1.4	24.7 ± 1.4	23.7 ± 1.9	29.8 ± 1.7	27.2 ± 2.3
Resting tremor	1.4 ± 0.2	1.3 ± 0.2	2.1 ± 0.3	2.0 ± 0.4	2.2 ± 0.5	2.0 ± 0.4
Bradykinesia	5.9 ± 3.5	5.5 ± 3.9	7.8 ± 0.6	7.9 ± 0.9	9.9 ± 0.7	9.1 ± 1.0
Rigidity	2.7 ± 0.2	2.5 ± 0.4	3.4 ± 0.3	3.0 ± 0.4	4.9 ± 0.5	4.7 ± 0.7
Postural reflex impairment	2.6 ± 0.2	2.8 ± 0.3	3.7 ± 0.4	3.7 ± 0.4	5.4 ± 0.6	4.9 ± 0.6
*p<0.05, based on genotype group comparisons using a t-test						

Table 7. Clinical characteristics of PEG PD patients of European ancestry, across follow-up exams by HLA *rs3129882* genotype (AA vs GG)

Data for clinical characteristics presented as mean +/- SEM. Follow-up exam 1 was a mean of 3.5 yrs after baseline (SEM=0.1) and exam 2, 5.6 yrs after baseline (SEM=0.2). In this table, subjects homozygous for the *rs3129882* SNP are compared in terms of

clinical disease characteristics over time. There is no difference in the clinical characteristics between the groups at any time point.

HLA-DRB1	**rho**	**p-value**	***HLA-DRB5***	**rho**	**p-value**
Utah Caucasians	0.199	0.038	**Utah Caucasians**	0.516	9.30E-09
Han Chinese	-0.281	0.0124	**Han Chinese**	-0.038	0.7396
Gujarati Indians in Houston, TX, USA	-0.017	0.8774	**Gujarati Indians in Houston, TX, USA**	0.242	0.0283
Japanese in Tokyo, Japan	0.038	0.7364	**Japanese in Tokyo, Japan**	-0.131	0.2393
Luhya in Kenya	-0.347	0.0014	**Luhya in Kenya**	-0.315	0.0039
Mexicans in Los Angeles, CA, USA	0.052	0.7333	**Mexicans in Los Angeles, CA, USA**	0.193	0.2029
Masaii in Kenya	-0.338	5.20E-05	**Masaii in Kenya**	-0.037	0.666
Yoruba in Nigeria	-0.317	8.00E-04	**Yoruba in Nigeria**	-0.122	0.2079

Table 8. The direction of association of cis-eQTL level with the *rs3129882* genotype depends on ethnicity

The level of HLA-DRB1 and HLA-DRB5 cis-eQTLs were significantly associated with *rs3129882* genotype and were within 500 kilobasepairs of the *rs3129882* SNP in the HapMap3 database. Association was reported as Spearman's rank correlation (rho) with p-value for all the ethnic groups within the database.

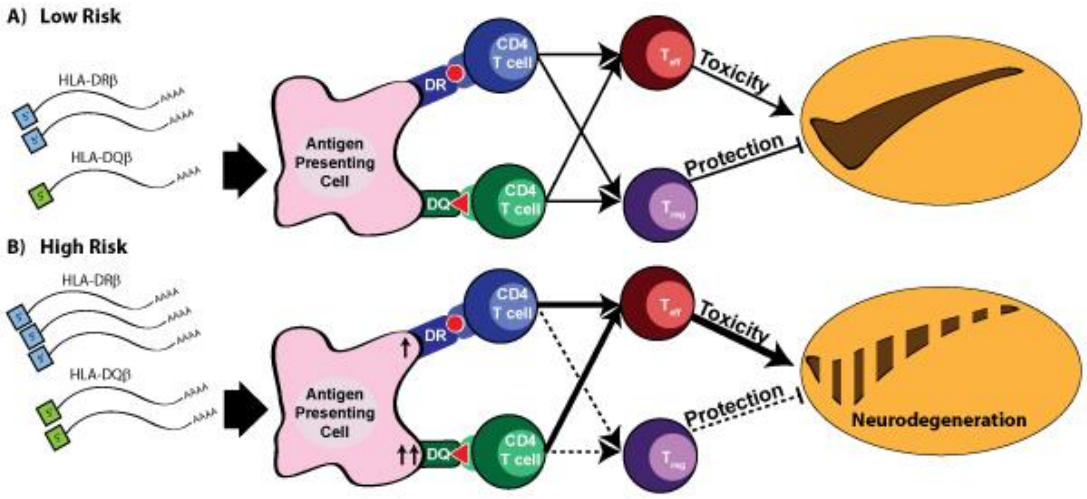

Fig 19. Model depicting the association of the *rs3129882* SNP with altered MHC-II expression on APCs and the potential for skewing the adaptive immune response and the predicted effects on vulnerable neuronal populations affected in PD. Our data suggest that the *rs3129882* SNP is linked to a genetic or epigenetic element that increases mRNA expression of MHC-II and ultimately surface expression of MHC-II. Increased surface expression of MHC-II can influence CD4+ T cell activation and differentiation leading to a heightened proinflammatory state that hastens neuronal dysfunction and death and predisposed individuals to PD. Two individuals exposed to the same environmental stimulus, one with the low risk SNP (A) and the other with the high risk SNP (B), are likely to respond differently immunologically because of underlying genetic and epigenetic mechanism(s) that influence MHC-II expression.

Chapter 5: The commonly used class of insecticides, pyrethroids, have acute immunomodulatory effects that impact mechanisms of antigen presentation

5.1) Introduction

Pyrethroids are a commonly used class of insecticides in the home and in agriculture. Developed for their neurotoxicity in insects, type I or II pyrethroids delay the opening of voltage-gated Nav1.8 and Nav1.9 sodium channels while type II pyrethroids can also decrease the opening probability of chloride ion channels and inhibit GABA-gated chloride channels[231]. Mammals express these same types of ion channels but acute exposure to these compounds in low doses is thought to be harmless to humans. Much of the animal work looking at these compounds has mainly demonstrated effects on neurodevelopment but their relevance on decreased neural function in adulthood remains controversial[232]. Recently, an unbiased epidemiological study implicated pyrethroids with antigen presentation through the *rs3129882* single nucleotide polymorphism (SNP) in the major histocompatibility complex (MHC)-II locus in synergizing to increase risk for Parkinson's disease[203]. The high risk SNP itself was shown to be associated with increased MHC-II expression and inducibility[203].

Parkinson's disease is a chronic, progressive neurodegenerative disease characterized by the loss of dopamine-producing (DA) neurons in the substantia nigra, neuronal inclusions called Lewy bodies, which are composed of α-synuclein and other proteins, and chronic neuroinflammation[48,49,199]. Exposure to pesticides such as paraquat and rotenone have been known to increase risk for PD[204,231]. Pyrethroid exposure has been shown in rodent models to alter striatal dopamine uptake and at very high doses induce DA neuron death[233-235]. It is well-known that genetic susceptibility and

environment synergize to create risk for PD but little is known about the mechanism behind how pyrethroids modulate the immune system to promote neurodegeneration[236].

Pyrethroids are known to induce caspase activation and apoptosis in immune cells in rodents to alter functions such as cytokine secretion and proliferation[237-239]. In humans, exposure to pyrethroids is associated with changes in serum cytokine levels and in levels of immune cells and complement proteins[225,240]. These changes remain within physiological levels but are significantly altered from baseline. Given this preliminary evidence for the effects of pyrethroids on immune function, we hypothesized that pyrethroids have effects on mechanisms that regulate antigen presentation to CD4+ T cells which may explain the epidemiological synergy between the *rs3129882* SNP and pyrethroid exposure. To activate CD4+ T cells, antigen presenting cells (APCs) must provide three signals: 1) presentation of antigen on the MHC-II molecule to the T-cell receptor, 2) expression of costimulatory molecules that bind receptors on T-cells that have recognized their cognate peptide:MHC-II molecule, and 3) cytokine secretion to instruct differentiation of T cells[114,116,223]. Upon activation, T cells proliferate and differentiate into discrete helper subsets to perform their effector functions, which include secretion of cytokines[114,116,223]. Herein, we investigated whether pyrethroids can alter these functions of APCs and T cells.

5.2) Materials and Methods

5.2a) Cell Culture

THP-1 cells (ATCC# TIB-202) and Jurkat cells (ATCC# TIB-152) were grown in culture using conditions from American Type Culture Collection (ATCC) guidelines. Cells were stimulated with 100 U/mL IFN-γ (Peprotech) or 100 ng/mL LPS (Sigma). For

THP-1 experiments, cells were first differentiated with 10 ng/mL PMA for 18h before treatment.

5.2b) RNA Isolation, cDNA synthesis, and RT-PCR

Cells were washed once in ice-cold phosphate-buffered saline and then lysed in 350 µL RLT buffer (Qiagen) supplemented with 1% β-mercaptoethanol (Sigma-Aldrich). Cell lysate was centrifuged through a Qiashredder (Qiagen) and then immediately frozen at -80°C. Later, RNA was fully isolated using RNAeasy Isolation Kit (Qiagen) and stored at -80°C. For cDNA synthesis, 0.5-2 µg of total mRNA was used per reaction in reverse transcription reactions using Superscript II (Life Technologies Corp) with oligo dT and random hexamer primers (Life Technologies Corp). The amount of SYBR-incorporated amplicons were measured for all real-time quantitative Bio-Rad iCycler instruments (Bio-Rad Laboratories) with an iQ optical module were used to measure the amount of SYBR incorporated amplicons for all real-time quantitative PCR reactions. DNA oligonucleotides (Integrated DNA Technologies) used for primers listed in **Table 2** were diluted to a final concentration of 100 nM for PCR reactions. All primers were tested by agarose gel electrophoresis to ensure that they formed single amplicon products of the correct size and optimized for Tm by temperature gradient real-time PCR followed by a melt curve analysis.

5.2c) Flow Cytometry

For surface staining, cells were washed with FACS buffer and staining for dead cell exclusion was performed on ice for 30 minutes using Fixable Red or Aqua dyes (Life Technologies). Cellls were then stained for 20 minutes with fluorophore-conjugated

antibodies. Antibodies used were anti-CD40-Pacific Blue (Biolegend), anti-ICOSL-PE (Biolegend), anti-CD86-PE-Cy7 (Biolegend), anti-CD80-APC (Biolegend), and Human Trustain FcX Receptor Blocker (Biolegend). Then, cells were washed and then fixed with 1% paraformaldehyde for 30 minutes. After washing, cells were stored in FACS buffer until analysis on a LSR-II flow cytometer (BD Biosciences). Data analysis was performed on FlowJo software.

5.2d) Multiplex Enzyme-linked Immunoassay

Conditioned media was analyzed for cytokines and (human IFN-γ, IL-1β, IL-6, IL-10, IL-8, IL-6, IL-12p70, IL-4, IL-3, and TNF) using a multiplexed immunoassay per the manufacturer's instructions (Meso-Scale Discovery, Gaithersburg, MD).

5.2e) Carboxyfluorescein Succinimidyl Ester (CFSE) Labeling

Cells were diluted in 1 mL of 1x phosphate-buffered saline at a concentration of at least 1 million cells per mL. Per manufacturer's instructions, CFSE (Life Technologies) was added at a staining concentration of 5 uM and incubated with cells at 37°C for 20 minutes. Cells were quenched with media for 5 minutes at room temperature before plating for assays.

5.2f) Pesticide Handling

Working stocks of rotenone, permethrin, and esfenvalerate (ChemService) were diluted to 1 mM in DMSO and stored at 4°C. Pesticides were diluted directly from working stocks into fresh cell culture media.

5.2g) Statistical Analyses

Comparison among more than two groups for experiments was analyzed by two-way ANOVA followed by the Sidak *post-hoc* test for *p* values. Comparison between just two groups was tested by the two-tailed Student's t-test. Specific statistical tests and number of replicates used in each group for every experiment are indicated in figure legends.

5.3) Results

5.3a) Rotenone and esfenvalerate alter induction of MHC-II mRNA in response to IFN-γ

The first signal for CD4+ T cell activation in antigen presentation is the engagement of the TCR with its cognate peptide:MHC-II molecule on the surface of the APC[114]. To test the effect of pyrethroids on MHC-II expression, we measured the effect of pesticides on MHC-II mRNA levels in unstimulated or IFNγ stimulated differentiated THP-1 macrophage-like cells by qRT-PCR. Rotenone was used as a non-pyrethroid pesticide control that also has known immunomodulatory effects. Concentrations for pesticides were chosen to ensure that less than 5% cell death occurred with treatment (data not shown). In unstimulated cells, pesticides did not alter MHC-II mRNA expression (Fig 20). Upon stimulation, rotenone was shown to dampen induction of *HLA-DRA* and *HLA-DRB* while enhancing *HLA-DQA* induction. Esfenvalerate enhanced induction of *HLA-DRB*, *-DQA,* and *-DQB* mRNA levels. Permethrin had no effect on the induction of the MHC-II genes measured. These results indicate that rotenone and esfenvalerate can both modulate the induction of MHC-II expression on cells of monocytic lineage.

5.3b) Pesticides dampen the induction of costimulatory molecules

The second signal for CD4+ T cell activation in antigen presentation is the engagement of costimulatory molecules on the APC cell surface. To test the effect of pyrethroids on APC expression of costimulatory molecule expression, we measured the effects of pesticides on baseline and LPS-stimulated levels of these molecules on differentiated THP-1 macrophage-like cells by flow cytometry. Again, pesticides did not have an effect on the baseline levels of cell surface costimulatory molecules (Fig 21). Rotenone dampened the induction of all cell-surface costimulatory molecules that were measured. Permethrin dampened the induction of CD80 and CD40 while esfenvalerate additionally dampened ICOSL induction. This experiment demonstrates that rotenone, permethrin, and pyrethroids dampen LPS-mediated induction of costimulatory molecules on cells of monocytic lineage.

5.3c) Pesticides dysregulate cytokine secretion

The third signal in CD4+ T cell activation is the cytokine milieu that directs the differentiation of T cells to perform their effector functions[114,116]. To test the hypothesis that pesticides alter cytokine secretion, we measured cytokine secretion in unstimulated or IFNγ stimulated differentiated THP-1 cells. The robust effects of rotenone were to significantly dampen TNF and IL-6 secretion (Fig 22). The robust effects of permethrin and esfenvalerate were to enhance IL-8 production while dampening TNF production in both unstimulated and stimulated cells. The levels of other measured cytokines were unchanged by pesticide exposure and/or IFNγ stimulation (data not shown). Thus,

pyrethroids dysregulate cytokine secretion from THP-1 cells while rotenone dampens cytokine secretion.

5.3d) Pesticides increase rate of T cell proliferation

The ultimate result of T cell activation is the licensing of T cells to perform their effector functions[114]. These T cell effector functions include T cell proliferation and cytokine secretion. To measure T cell proliferation in the presence of pesticides, CFSE-labeled Jurkat T cells were plated in the presence or absence of pesticides. After 60 hours, we ran these cells on a flow cytometer to measure the rate of cell proliferation by dye dilution. Cell proliferation was assessed by quantifying the frequency of cells that reached a second division (Fig 22A), by quantifying the fluorescence intensity of dye in the total cell population (Fig 22B), and by visually comparing the histograms of the dye content of the cell population (Fig 22C). Using all three parameters, it was apparent that permethrin and esfenvalerate significantly enhanced the rate of Jurkat T cell proliferation. Rotenone dampened T cell proliferation at higher concentrations but enhanced it at a low concentration. In brief, pyrethroids seem to enhance cellular proliferation, enhancing T cell effector function.

5.4) Discussion

Given the epidemiological synergy between the exposure to pyrethroids and the *rs3129882* SNP that increases MHC-II expression to increase risk for PD, this study seeks to understand the mechanism behind this interaction effect[203]. Using cell lines, we tested the effect of three different pesticides on the mechanisms of antigen presentation that provide signals to T cells. Permethrin and esfenvalerate are type 1 and 2 class

pyrethroids, respectively. Rotenone is an organophosphate pesticide associated with increased risk for PD with known immunomodulatory properties used in these assays as a positive control[57,205,241]. It was demonstrated that rotenone can alter MHC-II induction by IFNγ and that esfenvalerate can enhance it (Fig 20). Permethrin seemed to have no effect on the induction of MHC-II, which could be explained by potential of esfenvalerate to act on GABA-gated chloride channels as a type 2 class pyrethroid[231,232,242]. Furthermore, pyrethroids dampened LPS-induced expression of costimulatory molecules (Fig 21), dysregulated cytokine secretion (Fig 22), and enhanced T cell proliferation (Fig 23). These results demonstrate that pyrethroids are clearly immunomodulatory of mechanisms important for antigen presentation. Since rotenone also has immunomodulatory effects on processes relevant for antigen presentation, it may also be able to increase risk for PD in a similar manner to pyrethroids. Further epidemiological studies would be needed to support this hypothesis.

These data provide preliminary evidence for further investigation of how pyrethroids alter antigen presentation. All of this work was performed in cell lines and the evidence for these effects would be strengthened by confirmation in primary immune cells. Furthermore, because these results do not demonstrate clear effects in one direction but rather a complex dysregulation of immune responses *in vitro;* the type of dysregulated immune response that pyrethroids will create must be investigated using *in vivo* or *ex vivo* models of antigen presentation. It is known that the level of costimulation and cytokine stimulation alters the way in which CD4+ T cells differentiate but the aggregate effect of these signals is not well understood[114,116]. The Nav1.8, Nav1.9, and GABA-gated chloride channels can be detected at the mRNA in level in human immune

cells but confirmation of surface expression and the ability of pyrethroids to interact with these channels on immune cells is needed[243]. These cell-based studies serve to advance our understanding of the molecular mechanisms underlying the gene-environment interaction between rs3129882 and pyrethroid exposure that increases risk for PD and together these observations provide important clues regarding how the adaptive immune system responds to environmental exposures to modulate risk for PD. Further studies to understand the mechanistic effects of pyrethroids on inflammation should enable development of immunologic-based therapies with the potential to prevent, attenuate or delay the development or course of sporadic PD.

5.5) Figures

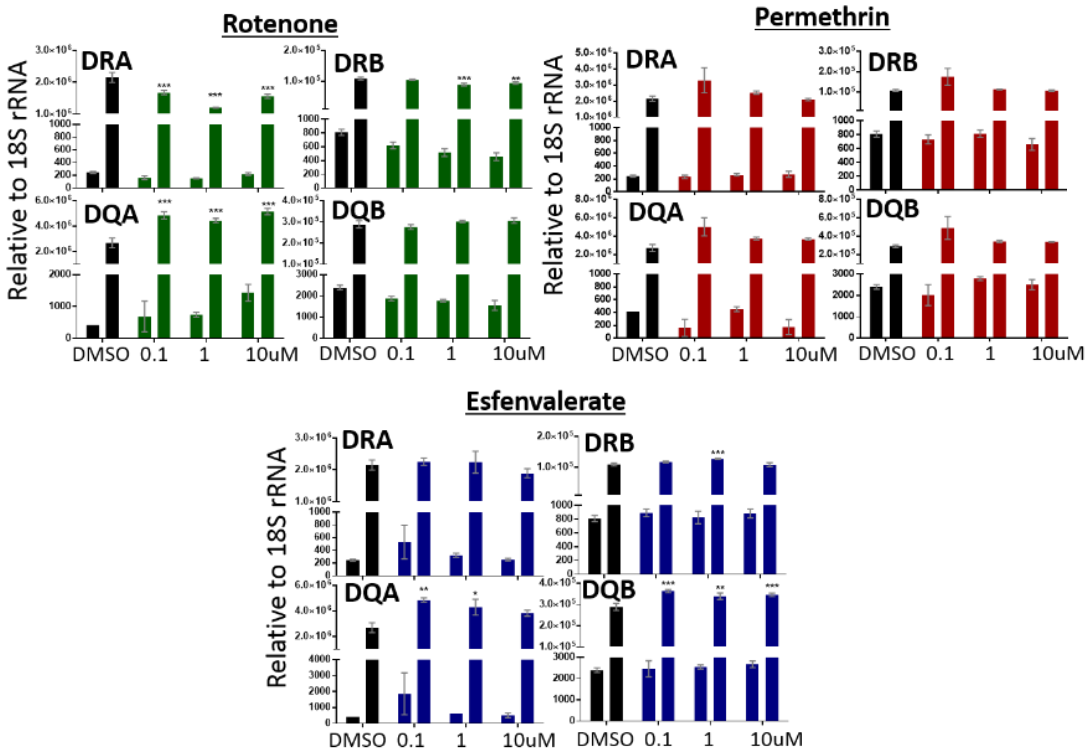

Figure 20. Esfenvalerate and rotenone but not permethrin modulate the IFN-γ mediated induction of MHC-II in THP-1 cells. THP-1 cells were differentiated

for 18h with 10 ng/mL PMA and then were either allowed to rest or were stimulated with 100 U/mL IFNγ in the presence of indicated concentrations of pesticides in quadruplicate. After an additional 18h, cells were lysed and RNA was isolated. MHC-II mRNA levels were assessed by qRT-PCR and data is expressed relative to 18S rRNA. Data is plotted as paired bars with unstimulated (left) and stimulated (right). Error bars represent SEM. Two-way ANOVA with Sidak *post-hoc* test was used to assess significance with treatment and stimulation as covariates. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

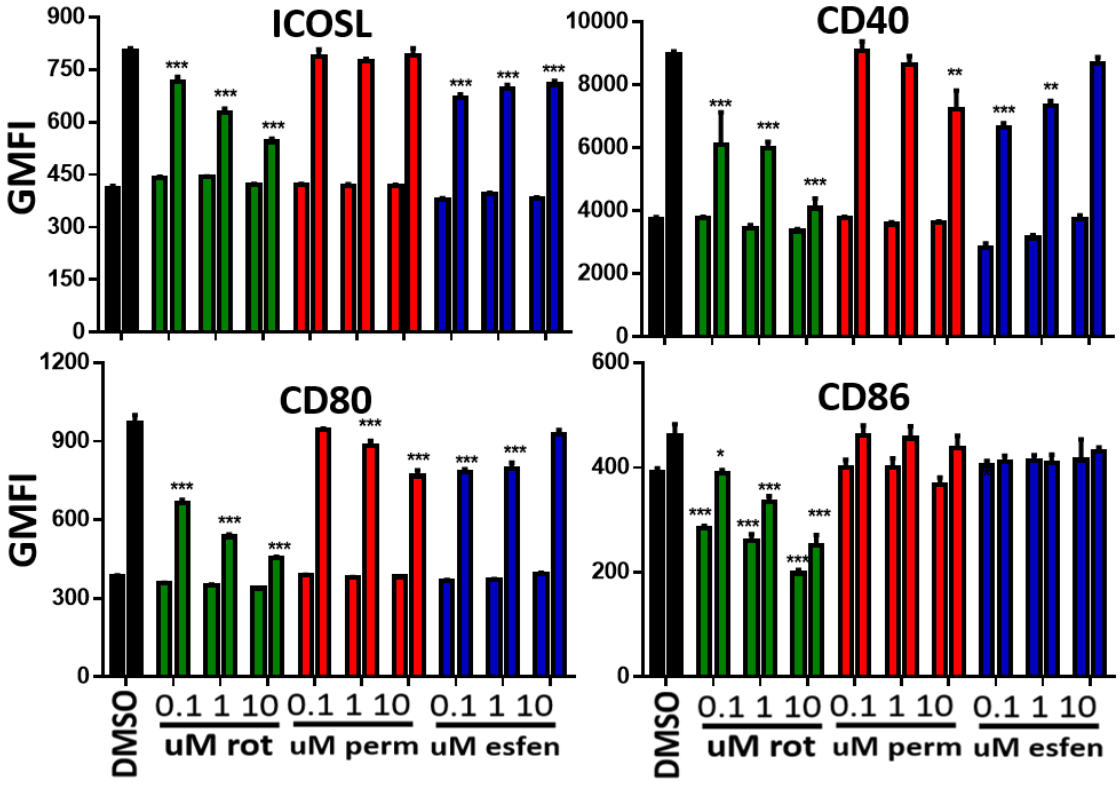

Figure 21. Pyrethroids dampen the LPS-induced expression of costimulatory molecules on the surface of THP-1 cells. THP-1 cells were differentiated for 18h with 10 ng/mL PMA and then were either allowed to rest or were stimulated with 100 ng/mL LPS in the presence of indicated concentrations of pesticides in quadruplicate. After an additional 18h, cells were harvested to evaluate surface expression of indicated

costimulatory molecules by flow cytometry. Data is plotted as paired bars with unstimulated (left) and stimulated (right). Error bars represent SEM. Two-way ANOVA with Sidak *post-hoc* test was used to assess significance with treatment and stimulation as covariates. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

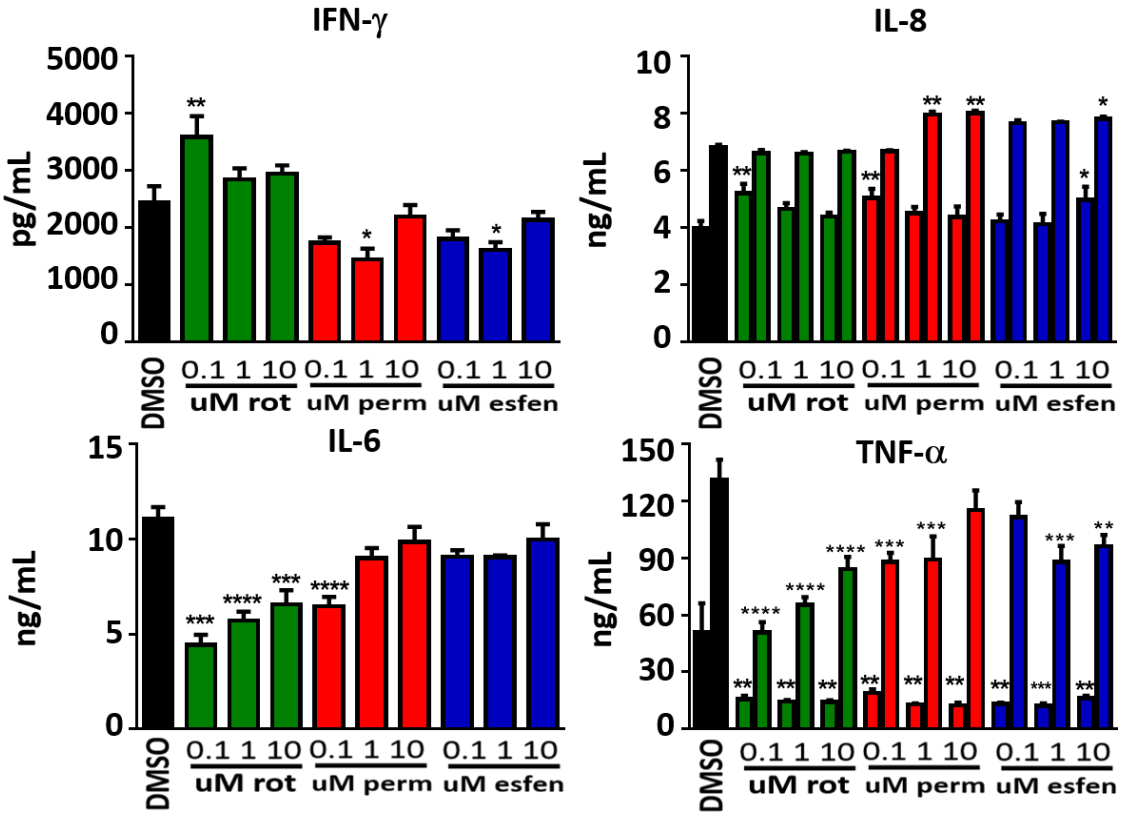

Figure 22. Pyrethroids alter the IFNγ induced secretion of cytokine in THP-1 cells. THP-1 cells were differentiated for 18h with 10 ng/mL PMA and then were either allowed to rest or were stimulated with 100 U/mL IFNγ in the presence of indicated concentrations of pesticides in quadruplicate. After an additional 18h, supernatant was collected for assessment of cytokine levels. When data is plotted with paired bars, they represent unstimulated (left) and stimulated (right). Unpaired bars represent the stimulated levels as unstimulated levels of cytokines were undetectable. Error bars

represent SEM. Two-way ANOVA with Sidak *post-hoc* test was used to assess significance with treatment and stimulation as covariates. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

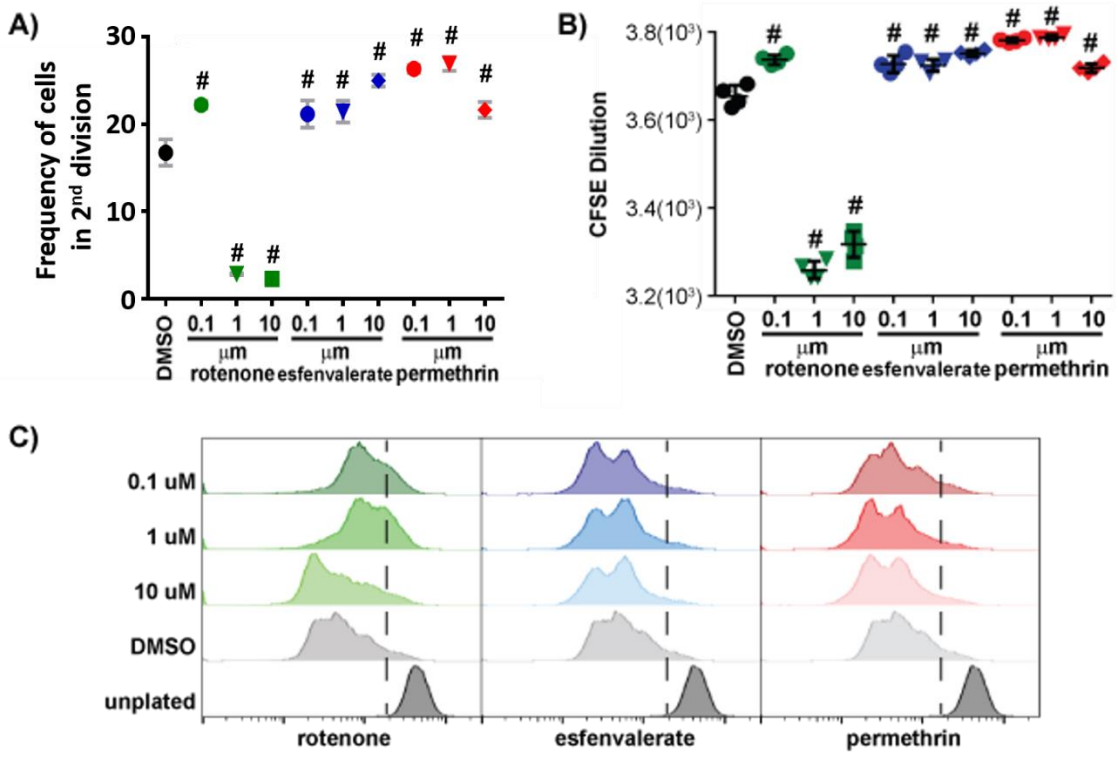

Figure 23. Pyrethroids increase rate of Jurkat T cell line proliferation. CFSE-labeled Jurkat T cells were allowed to proliferate in the presence of various concentrations of pyrethroids for 60h and then were analyzed by flow cytometry in quadruplicate. Frequency of cells in 3rd division (A), change in median fluorescence intensity 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. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Chapter 6: Future directions and applications to human disease and therapy
Incorporates previously published work[1].

6.1) Introduction

The ultimate goal of biomedical research is to advance the understanding of biological processes for the development of novel treatments for human disease. Given the findings of my studies on age-related changes in RGS10 expression in immune cells and its role in modulating immune cell chemotaxis, RGS10 could be a potential therapeutic target in neuroinflammatory conditions, particularly those that are age-related (Ch 2, 3). Chemotaxis regulates the trafficking of immune cells during normal homeostasis as well as sites of inflammation in disease[7]. The subtle changes that the RGS10-/- animals have in *in vivo* assays suggest RGS10 would be a good target to modulate immune chemotaxis without large immunocompromising side effects (Fig 12, 13). RGS10 expression in human immune cells resembles the relative distribution in mouse cells and decreases with age in CD8+ T cells and CD16+ monocytes in Parkinson's disease patients demonstrating human disease relevance (Fig 14). Further work is needed to determine how RGS10 should be targeted. RGS10-/- mice demonstrate a variety of phenotypes, including increased proinflammatory microglia and macrophages, as well as increased susceptibility to neurodegeneration[30,46,47]. However, in acute models of inflammation, the immune cell recruitment is largely unaltered in RGS10-/- mice with only specific cell subsets being impacted (Ch 3). Thus, RGS10 modulation may be important in currently untested models of chronic inflammation or in use to alter cell type-specific functions in disease.

The second part of this work demonstrated a role for the adaptive immune system in risk for Parkinson's disease (Ch 4). Pyrethroids synergize with antigen presentation to

increase risk for PD by modulating the processes of MHC-II expression, costimulation, and cytokine secretion (Ch 4,5). Pyrethroids are used commonly in household insecticide agents and in pest control operations[217,240]. Given that the *rs3129882* is a common variant and the use of pyrethroids allow for opportunity to repeated low-dose exposure, multiple exposures over a lifetime in the context of certain genetic backgrounds would put people at risk for PD. Outside of using public health measures to prevent pyrethroid exposure in humans, this work suggests a role for immunomodulatory therapies in the early stages of PD. The rest of this chapter illustrates future studies to extend the work described above and seeks to explore how RGS proteins and adaptive immunity could be targeted for immunomodulatory therapies in age-related neurodegenerative diseases.

6.2) Future Directions

In this work, RGS10 has been implicated in regulation of immune cell populations in aging as well as chemotaxis. To strengthen the association of RGS10 with chemokine signaling, assessment of additional downstream effector functions of chemokine activation should be determined in RGS10-/- versus WT cells. These effector functions include integrin activation and static adhesion. The modulation of these pathways by lack of RGS10 could be confirmed biochemically analyzing activation of proteins in the Vav-Rac pathway downstream of chemokine receptors. The mechanism by which RGS10 regulates these pathways could be investigated using GAP-dead mutants or by mutation of sites on RGS10 that determine covalent modification and in turn, cellular localization. In addition, rescue of the RGS10-/- phenotype by transfection of WT RGS10 would confirm the specificity of the findings of these studies. Translation of these findings into human cells could be achieved by assessing whether knockdown or overexpression of RGS10 in human PBMCs alters their chemotactic functions. Taken together, these findings would directly implicate RGS10 as a regulator of specific chemokine pathways. And To link these findings to age-related disease, altered chemotaxis in cells from aged RGS10-/- versus WT mice could be assessed to determine

whether chemotaxis defects regulate the age-dependent changes in immune cell populations reported in this work. From that point, one would be well-informed to pick better disease models in which to test the RGS10-/- animal and determine where RGS10-targeting therapeutics could be poised to be most helpful.

With respect to *rs3129882* and its impact on antigen presentation, two large questions remain. The first question is the mechanism behind the association of the SNP with increased expression and inducibility of MHC-II. In collaboration with the Boss lab, studies to assess epigenetic changes in association with rs3129882 are underway. No genetic or MHC-II haplotype associations have been identified suggesting an epigenetic mechanism[162,208,210,244]. The second question is the impact of increased MHC-II expression on neurodegeneration. To answer this question, studies are currently underway using mice with conditional myeloid knockout of MHC-II. Global knockout of MHC-II results in protection in a model of PD but that model also lacks CD4+ T cells in contrast to the conditional knockout model, which has normal numbers of circulating T cells[174]. A MHC-II overexpressing mouse would model the situation in humans with the high-risk SNP and would be expected to have enhanced neurodegeneration in a model of PD. Finally, To extend the findings looking at how pyrethroids interact with the antigen presentation process, a few experimental studies can be undertaken. First, cytokine secretion from Jurkat T cells should be assessed in the presence or absence of pyrethroids. Then, the cell line phenotypes should be confirmed in primary human PBMCs. The ultimate effect of the immunomodulatory effects of pyrethroids on antigen presentation can be tested *in vivo* in rodent models of vaccination or *ex vivo* with memory T cell responses to pools of viral peptides or to PD-relevant antigens like α-synuclein. Finally, the pyrethroid mechanism of action in immune cells needs to be confirmed through assessment of Nav1.8 and Nav1.9 sodium channels on these cells as well as the ability of pyrethroids to dampen sodium flux through these channels. The understanding

of the mechanism that links the *rs3129882* SNP to pyrethroid exposure would allow us great insight into developing therapies for PD and reducing risk for disease onset.

6.3) Immunomodulatory Therapies Targeting RGS proteins

Currently, there are no drugs approved for human use that directly target RGS proteins and few inhibitors or enhancers have been identified in preclinical models[245]. The main challenges with targeting RGS proteins is that there are few regions that are unique to particular subtypes of RGS proteins and these regions are highly sterically constrained; but allosteric modulators that bind on the side of the molecule opposite the active site have been identified[246]. Genetic models have been used to identify specific RGS proteins to target in various diseases. Loss of RGS4 function in mice showed a baseline "anti-depressed" phenotype by behavioral tests such as tail suspension and forced swim tests[247]. RGS4-/- mice also demonstrated reduced insulin resistance in a muscarinic M3 receptor activation model. Enhancement of RGS protein function could also be useful[248]. RGS2-/- mice are hypertensive and develop heart failure[14,249]. Interestingly, restoring RGS2 in the kidney was enough to reverse the hypertensive phenotype of the mice[250]. RGS9 overexpression in the nucleus accumbens has been shown to be beneficial for models of levodopa-induced dyskinesia as well as addiction[251,252].

Given the evidence in various preclinical models for the potential utility of RGS, many groups are attempting to identify molecules that enhance or inhibit the function of RGS proteins[245]. Yeast-two hybrid screens, peptide libraries, time-resolved FRET, and flow cytometry protein interact assays are common ways in which these approaches are being undertaken[253-256]. The most current understanding of RGS inhibitors is that they bind in an allosteric pocket to alter conformation of the RGS-Gα interaction[257]. This site is near where covalent modifications such as palmitoylation or phosphorylation of RGS proteins occur endogenously[258,259]. Given its subtle effects *in vivo* demonstrated in this

work, RGS10 could be used for selectively targeted in certain diseases to modulate inflammation perhaps without large side effects. It will be interesting to determine whether RGS10 modulation would alter a preclinical model of disease. If such a disease model is identified, specific inhibitors of RGS10 could be developed for human use.

6.4) Immunomodulatory Therapies in PD

This work has demonstrated that antigen presentation is a key process in putting people at risk for PD. Taken together with the evidence for the large amount of neuroinflammation present in PD, targeting the immune system for treatment could prove to be very useful in modifying disease[77,86,97,100,117,199,200,260]. Given the chronic, progressive nature of PD and the large amount of neurodegeneration that has already occurred before clinical presentation, it is unlikely that disease-modifying therapies will have a significant impact when applied late in the course of disease. Preclinical diagnosis and timely treatment will be necessary to significantly impact disease. Nevertheless, because intense investigation into biomarkers of early disease is in progress, it is critical that we continue identifying immunomodulatory therapies that could be translated to the clinic. To this end, there are a few therapies that target immune pathways have shown therapeutic benefit in animal models of PD.

One such example is that adoptive transfer of T cells from a copaxone-immunized animal showed protection against neurodegeneration in an MPTP model [261]. Copaxone, also known as glatiramer acetate, is used in the treatment of multiple sclerosis. It is a random polymer of amino acids from myelin basic protein. This mixture is thought to mimic the antigenic properties of myelin basic protein but alter the T cell response to a more anti-inflammatory phenotype [262]. This study suggests that T cell skewing to dampen the adaptive immune response could potentially have a disease-modifying effect. The adoptively transferred T cells accumulate in the SN, suppress microglial activation, and are correlated with an increase in neurotrophic factors [263]. Adoptive transfer of T_{regs} also

attenuated neurodegeneration in an MPTP model [112]. Decreased effectiveness of regulatory T cells in their suppressive capacities and changes in the T cell compartment suggest that correcting phenotypic T cell changes in PD patients may provide a beneficial disease-modifying effect [133]. Indeed, Dr. Howard Gendelman is initiating a clinical trial to assess the efficacy of Sargramostim, a granulocyte-monocyte colony stimulating factor analog thought to promote the development of Tregs, in the treatment of PD. If successful, this trial could demonstrate the usefulness of immunomodulatory therapies in treating this disease [264].

The use of a dominant-negative inhibitor of TNFα (DN-TNF) in PD animal models suggests that anti-TNF biological agents may also be of clinical value to delay or attenuate nigral DA neuron degeneration. Administration of soluble DN-TNF via osmotic pump or lentiviral overexpression in the SN shows protection in 6-OHDA models [260,265]. Even delayed lentiviral overexpression of DN-TNF in the SN can be protective against progressive DA neuron loss [266]. As a way to dampen overall levels of inflammation, anti-TNF biological tools could be of therapeutic value.

Passive immunization in animal models with anti-α-synuclein antibodies have also been investigated as a potential mechanism for clearing accumulations of α-synuclein. In mice expressing human α-synuclein under the platelet-derived growth factor β promoter, administration of an anti-α-synuclein antibody improves behavioral performance and promoted degradation of accumulated α-synuclein [267,268]. Clearance of α-synuclein is thought to occur via uptake through Fc-receptor expressing cells such as microglia. Whether clearance of α-synuclein-containing Lewy bodies would ameliorate PD symptoms, is an important question to pursue such a therapy. These observations will need to be replicated using other human α-synuclein overexpression models independently to confirm the promising potential of synuclein immunotherapy.
Since inflammation contributes to Parkinson's disease risk and pathology, the use of animal models to understand disease pathogenesis should be carefully considered.

Clearly any model should involve a-synuclein pathology given this protein's ubiquitous presence in PD neuropathology. Another critical component of any model should be a prominent inflammatory reaction that promotes neurodegeneration. Direct neurotoxin models mainly involve neuronal death without a significant contribution from inflammation and should be used with caution given the expanding evidence for immunopathology in PD.

Given some of the pathogenic contributions of adaptive immunity in PD described in this review, a few other strategies could potentially be used. These therapeutics would need validation or testing in animal models of PD. Therapies that block T cell proliferation may be useful in modifying progression or onset of PD. Such therapeutics could include corticosteroids, colchicine, or peripheral T-cell depleting antibodies such as muromonab-CD3. Colchicine blocks mitosis and thereby preventing the proliferation of rapidly dividing cells such as activated immune cells. Other alternatives or modifications of this drug may be needed as there are significant gastrointestinal side effects. In a rotenone rat PD model, colchicine showed benefit in preventing neurodegeneration [269]. Muronomab initially activates CD3+ T cells but then these activated cells die. This antibody is currently used in treatment of transplant rejection [270].

A second avenue is the use of drugs that block T cell activation or recruitment. Abatacept and belatacept block the interaction between CD28 on T cells with CD80/CD86 molecules on antigen presenting cells [270,271]. These drugs have been used in transplant rejection and rheumatoid arthritis with great success. Given the documented changes in activated and memory T cell subsets in people with PD, these drugs could have therapeutic success in limiting development of such T cell populations. The drug, natalizumab has been successful in the treatment of multiple sclerosis [272]. As an antibody against the α4 integrin, this antibody blocks the recruitment of T cells to sites of

inflammation. By blocking activation and recruitment of T cells in PD, neuroinflammation could be dampened to prevent subsequent neurodegeneration.

Ex vivo modification of autologous T cells to make them produce anti-inflammatory molecules or have them target effector T cells that are pathogenic in PD might also be another useful therapeutic approach. This approach would require identification of pathogenic T cell populations or the relevant antigens to which pathogenic T cells in PD respond. Such technologies are currently being explored in harnessing immune responses against cancer [273]. Some evidence exists implicating nitrated α-synuclein as a potential antigen in PD-relevant animal models [112,144]. The relevance of these experiments needs to be determined in human disease.

Therapies to target humoral immunity do exist. Rituximab is an example of a B cell depleting antibody that has shown some effectiveness in autoimmune diseases where antibodies are known to play a pathogenic role [274]. Intravenous immunoglobulin (IVIG) has been effective in treating diseases that are mediated by pathogenic antibody responses such as erythroblastosis fetalis, autoimmune thrombocytopenia, and chronic inflammatory demyelinating polyneuropathy. IVIG is thought to block proinflammatory antibody-mediated responses through many pathways including modulating Fc-receptor expression, expansion of T_{regs}, and blocking activating Fc-receptors [275]. IVIG is generally well tolerated with less than 5% of people experiencing headache, fever, or nausea. In a recent Phase III trial by Baxter, IVIG did not show efficacy in the treatment of mild to moderate Alzheimer's disease that may not reflect on its potential in PD since underlying pathogenic mechanisms are different. However, this study demonstrated that IVIG was safe in the elderly with neurodegenerative disease. Thus, IVIG could be a safe immunomodulatory therapy potentially useable for PD.

As with any immunomodulatory therapy, immunosuppression or off-target effects are of great concern, especially in an elderly population that is at increased susceptibility to infections. But as learned in the transplant field, timing of these and any other

therapeutic intervention is also critical [270]. Use of these therapeutics at the time where inflammation is beginning or is primed could have significant impact on the course of disease while having minimal effects much later in disease. Further understanding of the role of the adaptive immune system will help determine which avenues of immunomodulatory therapies are most likely to be effective.

6.4) Conclusions

As we better understand the mechanisms of how the immune system is regulated in the CNS, novel important biochemical pathways will be discovered. In this work, RGS10 and the adaptive immune system pose interesting targets for study and therapeutic intervention in neuroinflammation that were not previously recognized. In the years to come, mechanisms of inflammation will surely become an exciting target in treating neurodegenerative and neuroinflammatory conditions related to aging..

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