ABSTRACT

Hypertension afflicts nearly half of the adults in the United States and the majority of cases have no known cause. Chronic inflammation has been implicated in the development and maintenance of hypertension, and autoimmunity may comprise one of its sources. Hypertension is highly prevalent in the autoimmune disease systemic lupus erythematosus (SLE), in which chronic aberrant inflammation may be a causative factor. Endogenous neuroimmune pathways, such as the hypothalamic-pituitary-adrenal (HPA) axis and the cholinergic anti-inflammatory pathway, likely contribute to this phenomenon. The HPA axis is a classical neuroimmune mechanism that senses peripheral inflammation via afferent vagal fibers, culminating in the release of the anti-inflammatory hormone cortisol. Previous studies have characterized HPA axis dysfunction in SLE, but less is known about how this dysregulation specifically impacts the hypertension that occurs in the setting of SLE. A second neuroimmune interaction, the cholinergic anti-inflammatory pathway, is an efferent vagus nerve-to-spleen mechanism that relies on T cell-produced acetylcholine to quell inflammation in acute settings and may be hypoactive in chronic inflammatory diseases like SLE. Notably, both of these neuroimmune mechanisms depend on vagus nerve function, identifying the vagus as a potential target for neuromodulation. Furthermore, the relationship between chronic inflammation and hypertension validates the investigation of neuroimmune pathway dysfunction towards novel mechanisms of hypertension. Herewithin, the HPA axis and cholinergic anti-inflammatory pathway are investigated using the well-established *NZBWF1* mouse model of lupus hypertension. Our findings are that (1) administration of an inflammatory stimulus that activates vagal afferents elicits comparable neuronal activation in the paraventricular nucleus of the hypothalamus, compared to control mice, despite heightened peripheral inflammation; (2) amplification of efferent vagus nerve

activity reduces blood pressure and renal inflammation; and (3) chronic unilateral vagotomy paradoxically results in decreased blood pressure and renal inflammation. Taken together, these findings identify dysfunction in two neuroimmune pathways while demonstrating that interventions targeting these pathways may have therapeutic benefits in lupus hypertension. In terms of future impact, these results may promote continuing inquiry in a more recently discovered neuroimmune pathway (i.e., cholinergic anti-inflammatory pathway), as well as reinstate curiosity in an older, abandoned area of research (i.e., HPA).

DYSFUNCTIONAL NEUROIMMUNE PATHWAYS PROMOTE THE DEVELOPMENT AND MAINTENANCE OF LUPUS HYPERTENSION

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INTRODUCTION

Hypertension is clinically defined as a blood pressure of >130/80 mmHg measured on at least three separate occasions, and nearly 50% adults in the United States are hypertensive according to this definition (Whelton et al., 2018). This statistic alone is concerning, as hypertension is an independent risk factor for cardiovascular disease, the leading cause of death in America. Hypertension greatly increases the risk of stroke (Boehme, Esenwa, & Elkind, 2017), contributes to the development of heart failure (Messerli, Rimoldi, & Bangalore, 2017), and promotes the worsening of kidney disease (Tedla, Brar, Browne, & Brown, 2011). In terms of economic impact, the financial burden of hypertension alone was most recently estimated at $51.2 billion per year, with that of cardiovascular disease being $316.1 billion per year (G. Wang, Grosse, & Schooley, 2017). Despite advances in pharmaceutical treatment and experimental surgical techniques, the prevalence of treatment-resistant hypertension, defined as taking greater than three antihypertensive medications with continued uncontrolled hypertension, or taking greater than four antihypertensive medications overall, comprises almost 20% of adults in the United States (Carey et al., 2018). Currently, the most frequently prescribed antihypertensive medications target the adrenergic system (Michel, Brodde, & Insel, 1990), calcium channels on blood vessels (Muntwyler & Follath, 2001), and angiotensin-converting enzymes (Jackson & Bellamy, 2015). The persistence of hypertension in spite of contemporary medical management implicates other causes of elevated blood pressure that may not be sufficiently addressed by these current pharmacologic agents. Therefore, it remains necessary to explore other possible etiologies and potential therapeutic targets.

In recent decades, it has been shown that chronic inflammation contributes to the development and maintenance of hypertension. Chronic inflammation is an underlying factor in

many other diseases (e.g. autoimmune disease) and promotes end organ damage and in some instances, hypertension. Many patients with autoimmune diseases, such as systemic lupus erythematosus or psoriasis, also present with hypertension (Baghbanian, 2016; Panoulas et al., 2008; Rahman, Aguero, & Hallett, 2000; Salihbegovic et al., 2015), which is likely due to inflammation affecting the kidneys and other organs that regulate blood pressure. Inflammation is regulated in part by the nervous system, involving peripheral nerves, neurotransmitters, and humoral factors. Namely, the hypothalamic-pituitary-adrenal axis and the cholinergic anti-inflammatory pathway both comprise endogenous neuro-immune mechanisms that maintain homeostatic control of peripheral inflammation. However, these neuroimmune pathways have not been thoroughly investigated in the pathogenesis of hypertension. In addition, due to autonomic system aberration in patients with autoimmune diseases, autoimmunity may serve as a unique context in which neuroimmune aberrations leading to inflammatory causes of hypertension may be investigated.

Hypertension

In light of recent clinical guidelines, hypertension afflicts nearly 50% of adults in the United States (Whelton et al., 2018), thereby posing a major threat to human health and survival insofar as it is a major risk factor for cardiovascular disease, the leading cause of mortality in developed nations, such as the U.S. (Schroeder et al., 2003). Previously, hypertension had been defined as a blood pressure of >140/90 mmHg, with >130/80 mmHg being designated as pre-hypertension. Among hypertensive patients, approximately 90% of hypertension is deemed "essential," denoting that there is not an identifiable cause (Staessen, Wang, Bianchi, & Birkenhäger, 2003). By contrast, secondary hypertension has an attributable cause within the body and usually dissipates once this cause has been addressed, and common forms of secondary

hypertension arise from abnormalities within the renin-angiotensin-aldosterone system, hyperthyroidism, Cushing's syndrome (overproduction of cortisol), etc. (Berglund, Andersson, & Wilhelmsen, 1976). Treatment-resistant hypertension is a subdivision of essential hypertension that continues to persist despite the simultaneous administration of three or more anti-hypertensive agents (Pimenta & Calhoun, 2012). With hypertension being a multifactorial disease process, a multitude of possible etiologies have been investigated, including neural, renal, vascular, and metabolic causes. Current therapies include antihypertensive drugs (e.g. lisinopril [which inhibits angiotensin-converting enzyme], losartan [which blocks angiotensin II receptors], hydralazine [which mediates vasodilation]), neuromodulation (renal denervation) (S. Smith et al., 2016), and complementary and alternative medical treatments, such as acupuncture (Tam & Yiu, 1975), massage (Supa'at, Zakaria, Maskon, Aminuddin, & Nordin, 2013; Xiong, Li, & Zhang, 2015), meditation (Patel, 1977), and heat therapy. In the past decades, a plethora of human and animal studies have implicated the immune system in the development and maintenance of hypertension.

Overabundant inflammatory activity has been identified in human patients with essential hypertension, as indicated by elevated plasma concentrations of pro-inflammatory cytokines. Increased concentrations of the pro-inflammatory cytokine interleukin (IL)-1β were found in patients with essential hypertension prior to antihypertensive drug therapy (Dalekos, Elisaf, Papagalanis, Tzallas, & Siamopoulo, 1996). The pro-inflammatory cytokines IL-1β and IL-6 were shown to be increased following lipopolysaccharide challenge in blood sampled from essential hypertension patients compared to healthy controls (Peeters et al., 2001). Inflammatory markers, including C reactive protein, tumor necrosis factor (TNF)-α, and interleukin (IL)-6, were correlated with increased blood pressure in a cross-sectional study of a healthy Colombian

population (Bautista, Vera, Arenas, & Gamarra, 2005). A prospective cohort study conducted on a Dutch population also linked elevated C reactive protein with isolated systolic hypertension in older adults (Mattace-Raso, Verwoert, Hofman, & Witteman, 2010). However, there are no current therapies that specifically target the immune system and inflammation as a treatment for hypertension. This could be because immunosuppressive drugs do not uniformly result in decreases in blood pressure, despite antagonizing the pro-hypertensive actions of immune cells. Paradoxically, the immunosuppressive drugs tacrolimus and cyclosporine cause hypertension in patients who have received organ transplants (Canzanello Vincent J. et al., 1998; Hoorn et al., 2012). However, mycophenolate mofetil, which acts by selectively inhibiting purine synthesis in T and B cells (Ransom, 1995), has been demonstrated to mediate sustained decreases in blood pressure in patients with rheumatoid arthritis and psoriasis, as well as in patients with essential hypertension (Herrera, Ferrebuz, MacGregor, & Rodriguez-Iturbe, 2006). Therefore, there may eventually be a role for certain immunosuppressive and immunomodulatory agents in certain cases of resistant hypertension.

Animal studies have recapitulated findings gleaned from human hypertension studies, as well as revealed additional mechanisms by which immune cells mediate hypertensive injury. Chronic administration of cyclophosphamide, an immunosuppressive drug, prevents hypertension in spontaneously hypertensive rats (Khraibi, Norman, & Dzielak, 1984), an interesting parallel to the effects of mycophenolate mofetil on human hypertension. As in human hypertension, pro-inflammatory cytokines have also been implicated in experimental animal models of hypertension. Antagonists to IL-4 and IFN-γ, cytokines that promote T cell function, prevent hypertension in the *NZBWF1* mouse model of lupus hypertension (van Heuven-Nolsen et al., 1999). The production of IL-1β, IL-6, and TNF-α leads to atherosclerotic vascular injury and

hypertension in experimental chronic kidney disease in rats (Agharazii et al., 2014). In multiple studies, pro-inflammatory cytokine synthesis was found to be induced by locally-infused exogenous angiotensin II in wild type rats, resulting in hypertension and renal injury (Crowley & Thomas, 2014; Ruiz-Ortega et al., 2002; Zhang et al., 2014). The use of animal models provided further insight into the role of immune cells in hypertension, augmenting the pathophysiological narrative of cytokine and immunosuppressive treatment studies conducted in humans. Transplant of splenic and lymphoid cells from Lyon hypertensive rats to Lyon low-blood pressure rats conferred increases in blood pressure (Renaudin, Bataillard, & Sassard, 1995). Conversely, thymic nude mice, which lack a thymus and functioning T cells, are protected from experimental hypertension induced by deoxycorticosterone (DOCA) salt (Svendsen, 1976a), as well as renal infarction and contralateral nephrectomy (Svendsen, 1976b). T cells (Guzik et al., 2007) were later shown to specifically mediate angiotensin II-induced hypertension in rodents, as well as B cells, which are likewise required for antigen presentation to T cells (Chan et al., 2015). Renal infiltration of T cells is instrumental in the development of salt-sensitive hypertension (De Miguel, Lund, & Mattson, 2011; Hashmat et al., 2016; Mattson, 2014; Rudemiller, Lund, Jacob, Geurts, & Mattson, 2014). Additional studies identified novel compounds within immune cells that hinted at autoimmunity as a source of chronic inflammation leading up to hypertension. Acting as an initiator of immune cell activity, isoketals, compounds formed from lipid oxidation within dendritic cells, serve as a type of "self-antigen" to effector T and B cells (Kirabo et al., 2014). Most recently, the inflammasome, an endogenous immune apparatus that processes inflammatory cytokines (He, Hara, & Núñez, 2016), has emerged as a novel focus in research investigating chronic inflammation as an etiology for hypertension (D. Liu, Zeng, Li, Mehta, & Wang, 2017; Pasqua*, Pagliaro, Rocca, & Penna*, 2018). NLRP3 inflammasome functioning is

prominent to in human hypertension (Zhu et al., 2017), and inhibition of this inflammasome reduces blood pressure in experimental murine hypertension (Krishnan et al., 2018). In total, the role of the immune system in hypertension has been an intriguing focus of inquiry and will continue to be carefully delineated.

While chronic inflammation leading up to hypertension occurs systemically, there are specific and unique pathological changes that occur in the kidneys, which lead to chronic aberrations in renal physiology that promote abnormal blood pressure regulation. Inflammation, edema, and eventual fibrotic injury affecting the microvasculature of the renal glomeruli are responsible for a rightward shift and steepening of the pressure-natriuresis curve (Rodriguez-Iturbe & Johnson, 2010), as higher arterial pressures are increasingly needed to maintain urinary sodium excretion in light of this diminished local blood supply. The eventual loss of peritubular capillaries leads to local ischemia and the subsequent infiltration of T cells and macrophages into the interstitium. Renal T cells produce local angiotensin II (Hoch et al., 2009), which plays a major role in the renal pathophysiology leading to hypertension, in that it affects both glomerular function and renal inflammation; angiotensin II promotes glomerular vasoconstriction and augments proximal tubular sodium reabsorption, while directly increasing local TNF-α, IL-6, MCP-1, and NF-KB, which further recruit infiltrating immune cells (Ruiz-Ortega, Lorenzo, Suzuki, Rupérez, & Egido, 2001). The impact of inflammation in the kidneys thus mediates a vicious cycle wherein inflammatory injury diminishes renal microvasculature, leading to profound interstitial immune cell infiltration, and further pro-inflammatory cytokine-mediated injury. In the pathogenesis of hypertension, renal inflammatory injury leads to adverse outcomes in structural injury, as well as pathophysiological adaptations.

More specifically, chronic inflammation originating from autoimmune processes may be a pivotal force in the development and maintenance of specific forms of hypertension. Circulating IgG and IgM autoantibodies have been detected in patients with essential (Kristensen & Andersen, 1978) and pregnancy-related hypertension (Chan et al., 2014). In human patients, hypertension is prevalent in various rheumatic diseases, including psoriasis (Salihbegovic et al., 2015; Wu, Mills, & Bala, 2008), rheumatoid arthritis (Manavathongchai et al., 2013; Panoulas et al., 2008), multiple sclerosis (Baghbanian, 2016), and systemic lupus erythematosus (Herlitz, Edeno, Mulec, Westberg, & Aurell, 1984; Lozovoy et al., 2014; Mikdashi, Handwerger, Langenberg, Miller, & Kittner, 2007; Rahman et al., 2000; Ryan, 2009). In the case of SLE, inflammatory kidney injury or lupus nephritis is especially prevalent. Due to the increased detection of autoimmunity in hypertension, SLE and other rheumatic diseases may serve as a unique disease model in which hypertension may be investigated.

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) comprises an interesting model to study the process of chronic renal inflammation leading to hypertension and end organ damage. SLE was first identified by the 13th century physician Rogerius, who used the term "lupus," Latin for "wolf," to characterize the wolf bite-esque erosive facial lesions seen in the cutaneous form of the disease, which were red in nature ("erythro" being Greek for "red"). Later, SLE was recognized as a full-blown disseminated illness by Osler in the 1880s (Benedek, 2019). Currently, SLE is defined as a chronic autoimmune disease characterized by the inability of immune cells to recognize cells as self and instead regard them as foreign, leading to multi-systemic end organ damage, particularly

in the form of inflammatory kidney disease and cutaneous manifestations (Murphy, Lisnevskaia, & Isenberg, 2013). SLE predominantly affects women, with a nine-fold preponderance compared to men (Pons-Estel, Ugarte-Gil, & Alarcón, 2017). The origins and causes of SLE are not fully known, but a complex interplay of genetics and environmental factors culminate in an overactive immune system, wherein the immune system fails to develop tolerance to self-antigens. Autoimmune-mediated damage may occur in all organ systems, especially the kidneys, which gives rise to multiple patterns of the inflammatory kidney disease lupus nephritis. SLE is difficult to diagnose and requires identifying clinical signs and symptoms in addition to positive serologies for characteristic autoantibodies, including anti-double-stranded DNA (dsDNA), antinuclear (ANA), anti-Smith (Sm), anti-Ro, and anti-La, though clinical studies report varying specificity for the latter four (Dema & Charles, 2016). Most patients with SLE receive a delayed diagnosis due to the insidious and waning nature of the disease course, and typically experience initial nonspecific symptoms such as systemic joint pain, low-grade fever, and fatigue (Mosca et al., 2019). More worrisome and alarming symptoms of SLE include pleurisy (sharp pain induced by heavy breathing, due to inflammatory injury to the lining surrounding the lungs), hair loss, skin rashes, and severe lower extremity edema (swelling of the ankles) (Heinlen et al., 2007). In later stages of SLE, patients typically struggle with kidney failure, neuropsychiatric symptoms (Meszaros, Perl, & Faraone, 2012), and are at risk of strokes and thrombosis due to increased hypercoagulability (Boey et al., 1983). Treatment is aimed at preventing the progression of organ injury through courses of synthetic corticosteroids and other immunomodulatory drugs that target specific cytokine receptors, although specific immunosuppressive regimen may change following a disease flare (Michelle Petri, Genovese, Engle, & Hochberg, 1991).

Among SLE patients, the leading cause of mortality is cardiovascular disease, for which hypertension is a major independent risk factor, and indeed there is a high prevalence of hypertension among SLE patients. This identifies SLE as a unique disease process by which hypertension may be investigated amongst its backdrop of endogenous autoimmune over-activity. Hypertension is paradoxically common in young, reproductive-age women with SLE, and up to 50% of a given cohort may be hypertensive (Lozovoy et al., 2014; Oviasu, Hicks, & Cameron, 1991; Rahman et al., 2000; J. M. Sabio et al., 2011; Selzer et al., 2001). This is at odds with the general observation that this demographic is typically protected from hypertension, which is thought to be due to the vascular relaxing properties of estrogen (White, 2002). There are additional factors that may contribute to the high prevalence of hypertension in female SLE patients. Women with SLE have increased endothelial dysfunction compared to their disease-free counterparts (El-Magadmi et al., 2004), in addition to higher arterial stiffness (Brodszki, Bengtsson, & La, 2004; Selzer et al., 2001) and increased plasma homocysteine (M. Sabio et al., 2014), which have all been linked to the development of hypertension. While chronic inflammatory kidney disease is commonly comorbid with hypertension in SLE, it is not correct to attribute hypertension to pre-existing kidney disease. Lupus nephritis may occur in the absence of hypertension, and vice versa (Naiker, Chrystal, Randeree, & Seedat, 1997). However, uncontrolled hypertension worsens the prognosis of chronic kidney disease (Wadei & Textor, 2012; Ward & Studenski, 1992), making blood pressure control of special therapeutic importance in SLE (Tselios, Koumaras, Urowitz, & Gladman, 2014). Cardiovascular disease is also a major cause of mortality in SLE, with cerebrovascular accidents and myocardial infarctions occurring in the setting of hypercoagulable states (Kitagawa, Gotoh, Koto, & Okayasu, 1990; Manzi et al., 1997). Despite the presence of cohorts simultaneously afflicted by

both SLE and hypertension, it is difficult to elucidate possible mechanisms for lupus hypertension from human studies, as patients are typically maintained on a regimen of corticosteroids, which are known to provoke hypertension, in addition to various immunomodulatory agents (Chatham & Kimberly, 2001). Due to this important limitation, numerous rodent models of SLE have been developed in order to study mechanisms in the pathogenesis of SLE.

Various murine SLE strains, such as the NZM subtypes, BXSB/yaa, and MRL/lpr, have provided various insights into the development of SLE (Table 1) (Perry, Sang, Yin, Zheng, & Morel, 2011). However, female offspring of the New Zealand Black White First Generation cross (*NZBWF1*) are currently the only known SLE mouse model that becomes reliably hypertensive by a certain age (Rudofsky et al., 1984; Ryan, 2009). This is surprising, as every murine SLE model develops inflammatory kidney injury, or lupus nephritis, although it should be re-emphasized that there are also human SLE patients with lupus nephritis who are not hypertensive (Naiker et al., 1997). In the *NZBWF1* strain, there are sex differences that parallel those observed in human SLE patients, including later onset of SLE in males, as well as a greater prevalence of renal injury (Almeida, Arcoverde, Jacobino, & Neto, 2011; Dai et al., 2013). The autoimmune-induced hypertension observed in female *NZBWF1* mice is prevented by treatment with etanercept, a TNF-α blocker (Venegas-Pont et al., 2010), as well as by anti-CD20 antibodies that deplete B cells (Mathis, Wallace, et al., 2014), indicating that both T and B cells are implicated in the development of lupus hypertension. Likewise, treatment with mycophenolate mofetil, which inhibits T and B cell production, reduces blood pressure in this strain (Taylor & Ryan, 2017). In comparison to other experimental animal models of hypertension, the hypertension in the *NZBWF1* strain is not salt-sensitive (Mathis, Venegas-Pont,

Masterson, Wasson, & Ryan, 2011), at least at the stage of disease studied, but appears to be mediated in part by oxidative stress, as oral antioxidants were protective against hypertension in this strain (Mathis et al., 2012). Compared to the MRL/lpr and NZM strains, the *NZBWF1* strain takes a much longer time to develop SLE-like disease, but exhibits many of the hallmarks of human SLE, including the generation of characteristic anti-double stranded DNA (dsDNA) autoantibodies and lupus nephritis (Richard & Gilkeson, 2018).

Table 1. Characteristics of Murine SLE Strains

Murine SLE strain	Genetic Background	Disease Course	Predominant Symptoms	Unique Characteristics
NZBWF1	New Zealand Black x New Zealand White	Starts after 6 months of age, with 50% mortality in 9 month old females and in males at 15 months of age	Immune complex glomerulonephritis, mild vasculitis, splenomegaly, hypergammaglobulinemia, hypertension, cardiac lesions	Lacks rash and arthritis
NZM2328	NZB x NZWF1 for multiple generations. Sle1-3	Slower onset of disease with 50% mortality at 9 months, more prevalent in females	Two-stage renal disease: acute glomerulonephritis followed by a chronic nephritis	NZM2328 and other subtypes helped reveal specific disease-containing loci on *NZBWF1* genes
NZM2410	NZB x NZWF1 for multiple generations. Sle 1-3	Similar to NZM2328	Immune complex glomerulonephritis	Autoantibody production
BXSB/Yaa	Genetic risk mapped to the TLR7-containing Yaa locus on the Y sex chromosome	Disease only occurs in males	Immune complex glomerulonephritis	Only occurs in males

MRL+	Murphy Roths Large	Benign SLE disease course, starting at 18 months of age	Immune complex glomerulonephritis	Used primarily to study accelerants of SLE
MRL/lpr	Intercrossing of four different strains: LG, B6, AKR, C3H. lpr is a single-gene mutation in the Fas receptor gene	Accelerated course in females compared to NZBWF1, 50% mortality at 6 months	Immune complex glomerulonephritis, arthritis, cerebritis, skin rash, vasculitis, splenomegaly, lymphadenopathy	Full panel of SLE autoantibodies: ANA, anti-dsDNA, anti-Sm, anti-Ro, anti-La. Disease primarily driven by IFN-gamma instead of IFN-alpha, as in human SLE.
Pristane-induced	Created through intraperitoneal injections of pristane or other hydrocarbon compounds in BALB/c mice	More severe in females than males	Immune complex glomerulonephritis	Pristane induces autoantibodies characteristic of SLE (anti-Su, anti-Sm, anti-U1RNP, anti-dsDNA, and anti-histone)
Induced graft-versus-host disease	Parent into F1: lymphocytes transferred to a semiallogenic recipient	Severity correlated to the number of allografted cells, rapid disease onset	Immune complex glomerulonephritis	ANA, anti-dsDNA, anti-RBC autoantibodies

Dysautonomia in SLE

Glomerulonephritis, arthritis, and skin pathologies caused by prevailing chronic inflammation are certainly detrimental in SLE, but another debilitating, less obvious aspect of the disease concerns the autonomic nervous system. SLE patients commonly present with dysautonomia, the imbalance between activity of the parasympathetic and sympathetic nervous systems, measured through cardiovascular autonomic function tests, such orthostatic testing and cardiovascular responses to the Valsalva maneuver and the cold pressor test (Liote & Osterland,

1994; Shalimar, Deepak, Bhatia, Aggarwal, & Pandey, 2006). Since the 1980s, it has been well established that parasympathetic efferent vagus nerve activity, indirectly measured through heart rate variability on electrocardiogram, is dampened in patients with SLE (Aydemir et al., 2010; Thanou et al., 2016). Some investigators have proposed autoantibodies against autonomic nervous structures as the cause for dysautonomia in SLE (Maule et al., 1997). Conversely, autonomic sympathetic tone appears to be elevated in SLE (Capellino et al., 2008; Härle et al., 2006), and it has been well established that SLE disease flares are incited by psychological stress, which has a profound effect on the sympathetic nervous system (Herrmann, Scholmerich, & Straub, 2000). Additionally, following exposure to acute stress, the increase in natural killer (NK) cell population, as well as in β2 adrenergic receptor expression on circulating lymphocytes, was blunted in patients with SLE compared to their healthy counterparts, supporting an altered sympathetic-immune interaction in SLE (Pawlak et al., 1999).

The vagus nerve serves as a conduit between the brain and immune system, in that both the afferent and efferent fibers mediate anti-inflammatory neuroimmune pathways (Bonaz, Sinniger, & Pellissier, 2017). Vagal afferents activate the HPA axis by transmitting visceral information including peripheral inflammatory processes to the nucleus tractus solitarius. This nucleus tractus solitarius trigger leads to corticotropin-releasing factor (CRF) release from the paraventricular nucleus of the hypothalamus (PVN), adrenocorticotrophic hormone (ACTH) release into the bloodstream from the anterior pituitary, and cortisol or corticosterone release by the adrenal cortex. It is not certain how the efferent vagal fibers affect the HPA axis. The efferent vagus nerve initiates splenic cholinergic anti-inflammatory activity. Experimental therapeutic measures are directed towards increasing vagal tone and are achieved by pharmacological (Pham, Wang, & Mathis, 2018) or electrical means (Huston et al., 2007).

Presently, dysautonomia has not been well investigated in murine models of lupus, though there are indications that autonomic nervous system aberration would be present in murine lupus. The *NZBWF1* strain, which is considered the gold standard for studying cardiac SLE (Sanghera et al., 2019), has been shown to exhibit epicardial and myocardial lesions containing giant cells from as early as 4 months of age, which may variably affect nerve conduction. Additionally, the MRL/lpr murine lupus strain is known to exhibit neuropsychiatric manifestations of SLE in part due to dysfunction of the TWEAK/Fn14 pathway, which is responsible for mediating cytokine release by astrocytes and endothelial cells in the cerebral vasculature (Wen et al., 2013). It would not be unusual for dysautonomia to accompany neuropsychiatric SLE in murine strains, due to the heterogeneity of inflammatory brain lesions. Ultimately, there is a dearth of studies that have investigated heart rate variability and other functions dependent upon the autonomic nervous system in murine SLE. Further inquiry into this area of pathophysiology would be highly valuable, given the plethora of human studies that correlate autonomic nervous system activity to disease severity.

Neuroimmune Pathway Dysfunction in SLE

The nervous system is responsible for regulating a diverse array of bodily functions, from cardiovascular to digestive, but amongst its roles is the ability to regulate the immune system on a chronic basis. Afferent nerves transmit information regarding peripheral inflammation to the brain, which then elicits the release of neurotransmitters and humoral factors that modulate inflammation appropriately (T Hosoi, Okuma, & Nomura, 2000). The efferent arm of this nervous inflammatory reflex includes the hypothalamic-pituitary-adrenal axis and the

sympathetic nervous system, which both modulate peripheral immune activity. The cholinergic anti-inflammatory pathway is another pathway described within the last 20 years that has been demonstrated to attenuate inflammation in acute, experimental settings. While it is known that these neuroimmune pathways participate in maintaining homeostasis in healthy individuals, there are a lot of questions as to how they may be dysfunctional in the setting of chronic inflammatory disease. With autonomic system imbalance being a common feature of SLE, as indicated by dampened efferent vagal tone in the form of decreased heart rate variability, it is likely that neuroimmune pathways may be dysfunctional in the setting of SLE, and may contribute to chronic inflammation in the pathogenesis of hypertension in this disease process.

Aside from the HPA axis and the cholinergic anti-inflammatory pathway, there are other neuroimmune pathways that chronically regulate peripheral inflammation in the body and may likewise exhibit dysfunction in the setting of SLE. The sympathetic branch of the autonomic nervous system functions as a major endogenous neuroimmune pathway, as it may directly promote adaptive immune system activity through adrenergic receptors present on immune cells (Lubahn, Lorton, Schaller, Sweeney, & Bellinger, 2014). Some have proposed that the sympathetic nervous system directly activates the splenic nerve to release norepinephrine to immune cells in the sympathetic splenic neuroimmune pathway (Bellinger & Lorton, 2018). The splanchnic sympathetic nerves present in abdominal organs may also elicit the anti-inflammatory response in the setting of endotoxin administration (Martelli, Farmer, McKinley, Yao, & McAllen, 2018). The renal sympathetic nerves have been shown to contribute to renal inflammation in the setting of hypertension (Asirvatham-Jeyaraj et al., 2016; Xiao et al., 2015). Additional neuroimmune pathways include the auricular vagus nerve (Kaniusas et al., 2019; Zhao et al., 2012), as well as the PINK1- and PARK2-dependent pathways in sepsis (Kang et al.,

2016). The gateway reflexes (gravity, electric, pain, and stress) comprise means by which immune cells bypass the blood-brain barrier in order to induce neuroinflammation (Ohki, Kamimura, Arima, & Murakami, 2017). Microglia, astrocytes, and cells that line the blood-brain barrier have also been identified as entities that participate in neuro-immune communication (Maysinger et al., 2019). These pathways are not investigated in the scope of this dissertation, but are worthy of future inquiry regarding their role in lupus hypertension, as well as other chronic inflammatory diseases.

Hypothalamic-Pituitary-Adrenal Axis Dysfunction in SLE

The hypothalamic-pituitary-adrenal (HPA) axis is the most classically taught neuro-endocrine-immune pathway and mediates bodily responses to stress, inflammation, and glucose regulation. In the setting of psychological stress, the HPA axis is directly activated through central sympathetic mechanisms (Tsigos & Chrousos, 2002). The HPA axis can also be induced by input from vagal afferent fibers that have sensed circulating inflammatory markers (Mastorakos & Chrousos, 1994; Turnbull & Rivier, 1999) and bacterial products, such as lipopolysaccharide (LPS) (Johnston & Webster, 2009), which directly stimulates the innate system via toll-like receptor 4 and promotes the transcription of IL-1 and TNF-α by immune cells (Hoshino et al., 1999). LPS (T Hosoi et al., 2000) and IL-1 (Goehler, Gaykema, Hammack, Maier, & Watkins, 1998; Wieczorek & Dunn, 2006) have been shown to induce afferent vagus nerve firing that then initiates HPA axis activation, beginning with activation of parvocellular neurosecretory neurons in the paraventricular nuclei of the hypothalamus that release corticotropin releasing factor (CRF) into the portal system in the median eminence. CRF acts on

corticotrophs in the anterior pituitary that release adrenocorticotrophic hormone (ACTH) into the bloodstream, which binds to melanocortin 2 (MC2R) receptors in the zona fasciculata of the adrenal cortex and induces the adrenal release of cortisol (in humans) or corticosterone (in rodents). Cortisol is a pleiotropic steroid hormone with multiple effects: it stimulates gluconeogenesis, promotes sodium retention, and dampens immune system activity. Cortisol inhibits both innate (Chinenov & Rogatsky, 2007) and adaptive (Elenkov, 2004) immune processes. Specifically, it attenuates the inflammatory cascade (Chinenov & Rogatsky, 2007) and shifts the inflammatory milieu from a pro-inflammatory Th1 to an anti-inflammatory Th2 profile (Elenkov, 2004),. Cortisol and glucocorticoids may favor this shift to type 2-mediated anti-inflammatory responses by decreasing the generation of pro-inflammatory Th17 cells. Glucocorticoids also specifically down-regulate IL-1-mediated inflammatory events by increasing the concentrations of endogenous IL-1 receptor antagonist and IL-1 receptor II (Dujmovic et al., 2009). Unlike other autoimmune diseases that are characterized by prevalent increases in Th1 and Th17 cells, such as multiple sclerosis and rheumatoid arthritis (Raphael, Nalawade, Eagar, & Forsthuber, 2015), Th2-mediated events also participate in the development of SLE (Barcellini et al., 1996; Bedoya, Lam, Lau, & Larkin, 2013). Others have hypothesized that hypoactive HPA axis activity may predispose towards autoimmune diseases (Harbuz, Chover-Gonzalez, & Jessop, 2003), including SLE (Hu, Dietrich, Herold, Heinrich, & Wick, 1993; Lechner et al., 1996).

Others have demonstrated abnormal HPA axis responses to both inflammatory and non-inflammatory stressors in patients with SLE and mouse models, compared to their healthy counterparts (Gutiérrez, Garcia, Rodriguez, Rivero, & Jacobelli, 1998; Lechner et al., 1996; Rovenský et al., 1998). In light of these findings, it appears that the chronically heightened

peripheral inflammation in SLE may result from deficits in cortisol (or, in rodents, corticosterone) release. However, clinical studies have not demonstrably shown that baseline plasma cortisol is decreased in patients with SLE compared to their healthy counterparts. While some report decreased baseline cortisol (Alavi et al., 2003; Straub et al., 2004), others note comparable serum cortisol compared to healthy subjects (van der Goes et al., 2011). It is with regard to elicited cortisol responses that patients with SLE have been shown to differ compared to their disease-free counterparts. In response to insulin-induced hypoglycemia, there is an attenuated plasma cortisol or corticosterone response in SLE (Köller et al., 2004; Rovenský, Blazícková, et al., 1998). Others have shown that direct electrical stimulation of vagal afferents nerves which is known to activate the HPA axis (Toru Hosoi, Okuma, Matsuda, & Nomura, 2005), leads to interleukin (IL)-1β mRNA expression in the brain and increased plasma corticosterone (T Hosoi et al., 2000), supporting this connection. On the basis of this relationship, one might expect dysfunction of the vagal afferent-HPA axis interface in SLE, and this inquiry will be pursued in Chapter I.

An incomplete body of research has been conducted upon HPA axis dysfunction in SLE. One may initially hypothesize that the chronically heightened inflammation in SLE is due to deficits in baseline plasma cortisol, or insufficient cortisol release in the context of disease flares. However, clinical studies are mixed as to whether serum cortisol is inappropriately low or comparable to healthy subjects. One study found that serum cortisol was decreased in SLE patients compared to healthy subjects, though this was not due to increased renal clearance, increased conjugation, nor decreased adrenal production (Straub et al., 2004). A separate clinical study quantified serum cortisol in patients with SLE compared to healthy subjects as well as rheumatoid arthritis patients, showing that serum cortisol remained decreased an hour following

exercise induction (Alavi et al., 2003). Other studies indicate that patients with SLE have elevated plasma cortisol (Köller et al., 2004; Straub et al., 2004), although it is likely that SLE flares and disease severity impact HPA axis function. Clinical and basic science studies have both demonstrated that, in patients with SLE and mouse models of SLE (Gutiérrez et al., 1998; Lechner et al., 1996), the ability to raise plasma cortisol or corticosterone in response to an acute stressor is impaired compared to healthy counterparts. One in vitro study showed that lymphocytes isolated from patients with SLE catabolized cortisol at a greater rate than lymphocytes from healthy subjects, suggesting that immune system abnormalities in SLE may render normal plasma cortisol levels inadequate (Klein, A. Buskila, D. Gladman, D. Bruser, B. Malkin, 1990). In summary, there is not a clear explanation as to why baseline cortisol may be decreased in SLE patients compared to their healthy counterparts, nor as to why SLE patients and rodent SLE models have an attenuated cortisol or corticosterone response to an experimental stressor.

Exogenous glucocorticoids are a mainstay of clinical management of SLE (Chatham & Kimberly, 2001). However, usage of synthetic corticosteroids may contribute to further dampening of an already hypoactive HPA axis, due to negative feedback at the hypothalamus (Schlaghecke, Kornely, Santen, & Ridderskamp, 1992). Corticosteroid use is already known to have a slew of adverse side effects in SLE, from osteoporotic fractures to dyslipoproteinemia (Kasturi & Sammaritano, 2016). The vast majority of patients with SLE are maintained on exogenous glucocorticoids, which makes clinical studies on HPA axis dysfunction in SLE difficult to accomplish (C. Lee et al., 2005). Ideally, these studies involving disease mechanisms would be best carried out in patients with early-onset SLE, who may not yet be medicated. A clinical study showed that both basal and stimulated plasma ACTH and corticosterone did not

differ between untreated, new-onset SLE patients and healthy controls (Köller et al., 2004), suggesting that HPA axis dysfunction is a consequence, not an inciting cause, of disease progression. Cytokines have been shown to directly increase adrenal gland production of cortisol, independently of CRF (Bornstein, Rutkowski, & Vrezas, 2004), and since there is heightened peripheral inflammation in SLE, HPA axis dysfunction may further be compounded by cytokine-mediated adrenal gland activity. Additionally, exogenous glucocorticoids complicate clinical studies on hypertension in SLE because they cause sodium and fluid retention at the kidney and may increase blood pressure (Rahman et al., 2000). Therefore, animal studies designed to delineate links between SLE, the vagus nerve, the HPA axis, chronic renal inflammation, and hypertension would be ideal, as animals would not have the confounding effects of exogenous corticosteroids.

Alternative therapeutics related to the HPA axis have been indicated for the management of SLE. ACTH-containing gels are ideal for patients that cannot tolerate corticosteroids, and their mechanism of action is direct interaction with melanocortin 1-5 receptors (X. Li et al., 2016). ACTH, also referred to as corticotropin, was more commonly used in the 1950s for treatment of SLE flares, in conjunction with oral cortisone (Baehr & Soffer, 1950; Corcoran & Dustan, 1951; Harris-Jones, 1956). More recently, ACTH gels are being studied in clinical trials as an additional pharmaceutical treatment for steroid-dependent, resistant SLE (Furie et al., 2016). CRF is known to have direct anti-inflammatory properties against mast cells and macrophages (Audhya, Jain, & Hollander, 1991), but has not yet been utilized as a therapeutic agent in clinical trials.

Cholinergic Anti-Inflammatory Pathway Dysfunction in SLE

The cholinergic anti-inflammatory pathway is a more recently discovered endogenous neuroimmune pathway. The efferent vagus nerve, which terminates at the celiac ganglion, induces splenic nerve activation, prompting the release of norepinephrine into the spleen (L. V. Borovikova, Ivanova, Nardi, et al., 2000; Ji et al., 2014). This norepinephrine binds to β2-adrenergic receptors on a specialized population of T cells that are able to synthesize and release acetylcholine. This T-cell-derived acetylcholine then binds to α7nAChR on macrophages and other splenic immune cells, thereby inhibiting their production of pro-inflammatory cytokines (M. Rosas-Ballina et al., 2011). The cholinergic anti-inflammatory pathway has been identified in various models of experimental inflammation, from colitis (Ji et al., 2014) to obesity (X. Wang, Yang, Xue, & Shi, 2011), wherein disruptions of this pathway result in heightened inflammation, and interventions promoting activity of this pathway attenuate inflammation.

Recent studies have implicated possible cholinergic anti-inflammatory pathway dysfunction in SLE. Prior to its establishment, a plethora of clinical studies had demonstrated that efferent vagus nerve activity may be abnormally low in patients with SLE (CJ et al., 1997; Lagana' et al., 1996; Stein, McFarlane, Goldberg, & Ginzler, 1995; Thanou et al., 2016). This was evidenced by decreased heart rate variability in the high frequency power domain, which is a commonly accepted indirect measure of efferent vagus nerve tone. The ratio of high frequency (HF) to low frequency (LF) indices of heart rate variability, calculated from ECG tracings, has been proposed as a surrogate measure for the extent of peripheral inflammation, and has been correlated with active SLE in several clinical studies (Aydemir et al., 2010; Lagana' et al., 1996; Thanou et al., 2016). Female *NZBWF1* mice exhibit decreased changes in heart rate in response to atropine, an anti-muscarinic agent, likewise indicating impaired efferent vagal tone

(unpublished data). Interventions directed towards increasing cholinergic anti-inflammatory activity at various target sites in this mouse model of SLE have been demonstrated to improve blood pressure and improve other disease outcomes. Distally, at the level of the α7AChR in the spleen, subcutaneously-administered nicotine protected animals from hypertension and renal inflammation (Fairley & Mathis, 2017). Therapeutic interventions directed towards increasing efferent vagus nerve outflow may also improve blood pressure and end-organ injury in SLE. Taken together, these studies support the cholinergic anti-inflammatory pathway as having a therapeutic role in SLE and furthermore suggest that this endogenous neuroimmune pathway may be normally hypoactive in the disease process.

HYPOTHESIS AND SPECIFIC AIMS

Hypertension in the setting of SLE and other autoimmune diseases is likely impacted by dysfunction of endogenous neuroimmune pathways, and further inquiry into this niche is warranted for two reasons: (1) to better manage lupus hypertension, and (2) to gain additional insights into the role of the immune system and chronic, specifically renal, inflammation in the pathophysiology of essential hypertension. Future studies may focus on noninvasive means of augmenting neuroimmune pathway activity, which may decrease overall inflammation and disrupt the chronic cycle of vascular and end-organ injury that sustains hypertension.

The interaction between nervous and immune systems is a unique interplay that has been implicated in disease processes such as sepsis and ileus, but requires additional exploration in the setting of hypertension. I **hypothesize** that the HPA axis and the cholinergic anti-inflammatory pathway are dysregulated in a mouse model of SLE, and that these aberrant interactions contribute to the development and maintenance of hypertension in this mouse model of SLE.

In order to investigate this hypothesis, the following specific aims are proposed:

- Specific Aim 1: To establish that there is dysregulation of neuroimmune pathways in murine SLE (Chapters I, II, and III).
- Specific Aim 2: To demonstrate that interventions targeting the vagus nerve may be therapeutic in murine SLE (Chapters II and III).
- Specific Aim 3: To utilize natural compounds that target the vagus nerve in a translational study to yield beneficial outcomes (Chapter IV).

The first chapter investigates connections between vagal afferent nerves and the HPA axis by utilizing LPS challenge. The second chapter establishes that galantamine, a central cholinergic agonist, induces efferent vagus nerve activation and that chronic administration of this agent reduces blood pressure and renal injury in a mouse model of SLE. The third chapter demonstrates how cervical vagotomy may affect blood pressure and peripheral inflammation in a mouse model of SLE, as well as the rationale for the paradoxical findings that this intervention, when administered chronically, improves these parameters. The fourth and final chapter investigates the role of curcumin, an agent shown to activate vagal afferents via TRPV1 channels, as a means of chronically enhancing neuroimmune pathway activity in a mouse model of SLE.

GENERAL METHODS

Animal model: Female *NZBWF1* and *NZW/LacJ* mice were obtained from Jackson Laboratories (Bar Harbor, ME). The *NZBWF1* strain is generated by crossbreeding *NZB* female and *NZW* male mice. The parental *NZB* strain is notable for moderate autoimmunity throughout its lifespan, while the *NZW* strain presents with mild autoimmunity. Genetic studies have identified disease-modifying loci on chromosomes 4 and 12 for *NZB* and *NZW* mice, pertaining to glomerular cell damage, increased total IgG and IgM, for the former, and increased serum IgG anti-chromatin and anti-dsDNA autoantibodies, for the latter. The *NZBWF1* strain has a similar female preponderance as in human SLE, characterized by lymphadenopathy, splenomegaly, hypergammaglobulinemia (specifically with the production of anti-dsDNA autoantibodies), and immune complex glomerulonephritis, the latter of which becomes detectable around 5-6 months of age and typically progresses to severe kidney failure and death at 10-12 months of age (Perry et al., 2011). Unlike human SLE patients, the *NZBWF1* strain does not present with arthritis or skin lesions (Perry et al., 2011). Female *NZBWF1* mice have an average lifespan of 245 days, compared to 406 days for male mice.

As in previous studies (Fairley & Mathis, 2017; Mathis et al., 2012, 2011; Mathis, Wallace, et al., 2014; Venegas-Pont et al., 2011), *NZBWF1* mice that had an albuminuria of ≥300 mg/dL for two consecutive weeks, were used in the experiments featured in Chapter I, under the rationale that these mice with aggravated disease course would display the greatest amount of HPA axis dysfunction, and yield larger differences compared to the *NZW* strain. *NZW* mice are one of the parental strains of *NZWBF1* mice and exhibit mild autoimmunity, and were used as controls due to their similar lineage, yet lack of explicit SLE-like symptoms. Inclusion criteria for the studies conducted in Chapters II-IV were an albuminuria of ≤300 mg/dL for two consecutive weeks at

30 weeks of age, so as to prevent the use of mice with fulminant SLE, which would exhibit a severe and aggravated disease course regardless of the intervention utilized. All animal studies were approved by the University of North Texas Health Science Center's Institutional Animal Care and Use Committee (IACUC) and were in accordance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Routine Animal Procedures: Treatments were initiated when mice reached at least 30 weeks of age, in light of anticipating the development of severe renal injury at around 34-35 weeks of age. Mice were weighed on a weekly basis starting at 29 weeks of age and continuing throughout the study period. Urinary albumin was monitored weekly via dipstick (Multistix 10 sg 2161; Siemens; Sacramento, CA) throughout the study from overnight urine collection in metabolic cages in order to quickly and grossly evaluate the extent of renal injury (and by proxy, disease severity) for the purpose of determining inclusion criteria and monitor the morbidity of the mice. A more precise and accurate measure of albuminuria was later confirmed from urine collected at 30 weeks and at 34-35 weeks of age via ELISA (Cat. No. 1200; Alpha; San Antonio, TX). Anti-dsDNA autoantibody activity was also measured by commercial ELISA (Alpha) from plasma collected the start of the studies (30 weeks). In order to determine inclusion for mice using plasma collected at 30 weeks, the positive index was calculated from the net optical density of plasma from the *NZW* mice plus two standard deviations. The optical density of the same from each mouse was divided by this positive index; values greater than 1.0 denoted positive antibody activity (a requisite for *NZBWF1* mice and disqualifier for *NZW),* while values less than 1.0 denoted negative antibody activity (a requisite for *NZW* mice and disqualifier for *NZBWF1*). In order to verify that that the *NZW* included in the study had titers below two standard deviations of the average of *NZBWF1* mice could be included in the study results by having an anti-dsDNA

autoantibody titer greater than the threshold for *NZW* mice, and, and at the end of the studies (34-35 weeks), to assess the impact of the interventions used upon disease severity. Upon conclusion of studies, mice were euthanized by exposure to excess isoflurane, after which they received cervical dislocation.

Acute vagus nerve recording: Vagus nerve recordings were performed on anesthetized animals. SLE and control mice were weighed and intraperitoneally injected with α-chloralose (80 mg/kg) and urethane (1,000 mg/kg) to maintain an anesthetized and unconscious state throughout the entire duration of the recording session. Fur was shaved off the cervical region and mice were instrumented with a model SPR 671 Millar catheter in the left carotid artery to continuously record blood pressure and heart rate. The right cervical vagus nerve was localized and placed on a hooked platinum-iridium electrode (36 gauge). The electrode and nerve interface were encased in QuikSil silicone gel to minimize electrical noise and interference from the environment. Electric signals were amplified (×200,000) and filtered (band pass: 10–10,000 Hz) to isolate vagus nerve activity. A 10-min baseline was recorded in Spike2 (Cambridge Electronic Design), and then a single dose of galantamine (4 mg/kg dissolved in saline) was intraperitoneally injected, with the following peripheral vagus nerve activity recorded. Vagal activity was allowed to recover to baseline, and this process was repeated.

Chronic galantamine administration: At 32–33 wk of age, SLE and control mice were injected intraperitoneally with galantamine (4 $mg \cdot kg^{-1} \cdot day^{-1}$; Sigma, St. Louis, MO) (Pavlov et al., 2009) or the vehicle, normal saline, for 14 consecutive days. This dose of galantamine was found to potentiate central cholinergic activity and increase efferent vagus nerve activity in previous studies, though only in acute settings of experimental sepsis (Ji et al., 2014). Mice were continually monitored throughout the treatment period for signs of gastrointestinal side effects

(constipation, loss of appetite, weight loss) and neurological abnormalities (irregular gait, tremor) due to galantamine's effects on cholinergic transmission.

Direct Blood Pressure Measurements in Conscious Mice: Individual mice were anesthetized under 2% isoflurane in an anesthesia chamber, then laid prone with the head facing the animal surgeon. Commercially available depilatory ointment was used to remove hair on the ventral surface of the neck and upper chest area, and the bare skin was sterilized through at least three repeated cycles of betadine and ethanol application. An approximately 2-centimeter midline incision was made from the sternum towards the base of the neck. Under a dissecting microscope, the left carotid artery was carefully identified and isolated from the cervical vagus nerve via an upper and lower silk suture ligature. A catheter heat-stretched from Renapulse (Braintree Scientific) with a narrow 8 to 10mm narrow segment was inserted into the left carotid artery until the tip of the narrow segment reached the aortic arch, evidenced by pulsing of blood into the catheter. The end of the catheter was then tunneled through the left side of the neck via the base of the dorsal neck, with 1% heparinized saline used to flush blood until no longer visible, and sealed shut using heated forceps. After recovering on a heating pad for at least fifteen minutes, mice were monitored for an additional hour before being returned to their animal room in the Department of Laboratory Animal Medicine (DLAM) facilities. The next morning, mice were connected to blood pressure transducers, and 1.5 hours of data total were recorded of mean arterial pressure using PowerLab software, with the last hour used to calculate mean arterial pressure by using an in-program function to calculate this mean value from the cumulative arterial pressure tracings. Mice were awake and conscious during this recording time. Following blood pressure measurement, the catheters were flushed with 1% heparinized saline, then the ends of the catheters were heat-sealed so that the mice could return to their DLAM

facility room. A second set of blood pressure measurements were obtained the following morning, and then the animals were euthanized.

Autoantibody quantification: Plasma double-stranded DNA (dsDNA) autoantibodies, an index of SLE disease severity, were quantified via commercial ELISA kits (Alpha Diagnostics) according to manufacturer's instructions, and as previously described (Fairley & Mathis, 2017; Gilbert, Mathis, & Ryan, 2014; Mathis et al., 2013, 2012; Venegas-Pont et al., 2011). This kit was an indirect ELISA that utilized horseradish peroxidase for colorimetric detection and quantification of IgG1 anti-dsDNA autoantibody concentration. Plasma was collected via the retro-orbital sinus using 10G syringe needles dipped in ethylenediaminetetraacetic acid (EDTA). Individual mice were firmly grasped by the dorsal aspect of their neck, with traction placed on the right base of cranium to hold the skin near the right orbit taut. The retroorbital sinus, located approximately 2 mm caudal from the right orbit, was pricked with the 10G needle and 6-7 drops of blood were collected in 1 mL microcentrifuge tubes. An alternative method for blood collection was employed by using approximately 1cm long segments of glass capillary tubing, which were submerged in EDTA and then applied with firm pressure to the caudal corner of the right orbit, through which blood was transferred to microcentrifuge tubes. Microcentrifuge tubes were centrifuged at 4 degrees Celsius for 10 minutes at 10,000 G, and then the plasma was transferred using disposable pipettes into new microcentrifuge tubes.

Protein homogenization for western blotting: Upon euthanasia of anesthetized mice, the spleen was dissected from each mouse and was homogenized at 4°C in eight times the organ weight of RIPA buffer. The renal cortex was dissected from the right kidney and was also homogenized in RIPA buffer (eight times the tissue weight). Aortas were stripped from the spine of each mice and then flash frozen in liquid nitrogen to quickly crush with a plastic plunger to homogenize with an appropriate amount of RIPA buffer (i.e., eight times the weight of the aorta). Tissue homogenates were transferred to microcentrifuge tubes and spun at 10,000G at 4 degrees Celsius for 20 minutes. Supernatants were transferred to clean microcentrifuge tubes via disposable pipettes and stored at -20 degrees Celsius.

Bicinchoninic acid (BCA) protein assay: The Thermo Scientific Pierce BCA Protein Assay was used to determine protein concentrations of tissues. A set of 9 protein standards were mixed by diluting bovine serum albumin with deionized water to the following concentrations: 2000 ug/mL, 1500 ug/mL, 1000 ug/mL, 750 ug/mL, 500 ug/mL, 250 ug/mL, 125 ug/mL, 25 ug/mL, and 0 ug/mL. Working reagent was prepared according to kit instructions, utilizing 25 mL of kit solvent A to 500 uL of kit solvent B. The standard curve was then pipetted with 1 replicate to comprise the standard curve. 6 uL of each tissue homogenate was mixed with 54 uL of DI water in microcentrifuge tubes, then each sample was pipetted into the microplate with 1 replicate. 200 uL of the prepared working reagent was added to each well via multichannel pipette. The microplate was sealed with parafilm, shaken on a plate shaker for 30 seconds, and incubated at 37 degrees Celsius for 30 minutes. Afterwards, the plate was allowed to cool to room temperature, then the absorbance values for each well was read at 562 nm on a plate reader.

Western blotting: Standard Western blotting techniques were performed using splenic, cortex-rich renal, and aortic homogenates (Fairley & Mathis, 2017) to measure TNF-α (catalog no. sc-

52746; Santa Cruz Biotechnology, Santa Cruz, CA); high mobility group box protein 1 (HMGB-1) (catalog no. sc-26351; Santa Cruz Biotechnology); and BAFF (catalog no. MAB1357–100; R&D Systems, Minneapolis, MN), which are all well accepted markers of inflammation. Running buffer was prepared by diluting buffer 10:1 with running water to buffer concentrate. Gel electrophoresis chambers were filled up to designated level (approximately 400 mL per side) with reconstituted running buffer. Premade commercial 18 or 26-well gels (Bio-Rad) were removed from individual packages, then placed into electrophoresis apparatus. Additional running buffer was added to the top compartment of the plastic encasement of the prepared gel. Residual air was flushed from each well. 3 μL of Western C Standard Ladder concentrate was injected into well 1 of each gel. A volume containing 100ng of protein for each tissue homogenate was mixed with an equal volume of Laemmli sample buffer, beta-mercaptoethanol, in microcentrifuge tubes, sonic for 2 seconds, then heated for 10 minutes at 37 degrees Celsius in a water bath. The homogenate solutions were pulse vortexed again, then spun at 10,000G for 1 minute at room temperature. The entirety of each tube was loaded into wells. The electrophoresis chamber was connected to the power supply, set to 1.5 AMP and 250 volts. Total run time was around 25 to 27 minutes. The plastic casing was then deconstructed so that the intact gel could be transferred to a clear membrane case lined with approximately 10 mL of the reconstituted running buffer. The gel was transferred to the ChemiDoc MP Imaging System (Bio-Rad Laboratories; Hercules, CA) glass screen for "Gel Activation," which uses UV light to further disrupt methyl bonds in the protein bands for 1 minute of total activation time. The protein bands were transferred to a nitrocellulose membrane by creating a transfer sandwich and using the BioRad TurboTransfer apparatus, which applied 1.7A and 25V over 5 minutes. To verify that the protein bands had transferred completely, the gel and membrane were each imaged on the

ChemiDoc. The membrane was returned to a clear container filled with 30 mL of 10% blocking buffer (1.5 grams of powdered milk powder to 30 mL of TTBS) and allowed to incubate at room temperature for 1 hour on a rocking platform. Following this blocking process, the blocking buffer was mixed with 30 mL Tween 20 and the primary antibody of interest, then returned to the clear container, in which the membrane incubated overnight at 4 degrees Celsius. The following day, the membrane was washed three times, five minutes each wash, with approximately 10 mL of TTBS at room temperature on a rocking platform. The membrane was then incubated at room temperature for 1 hour on a rocking platform with 30 mL of blocking buffer mixed with a secondary antibody compatible with the primary antibody and 20uL of Tween 20. Following incubation with the secondary antibody, the membrane was washed three times with TTBS, then imaged on the ChemiDoc to obtain a stain-free image. The membrane was incubated in a light-free space with 10 mL of the Clarity Western ECL (Bio-Rad) for 1 minute, then imaged on the ChemiDoc with the same placement as the stain-free image capture. The composite stain-free and post-ECL images were analyzed using Image Lab software version 5.1. Data are reported as volume intensity, which refers to the cytokine densitometry of the protein band normalized to total lane protein, which was determined by the stain-free total protein method as previously described (Gilda & Gomes, 2013). For TNF-α, both the 26- and 51-kDa bands were analyzed, the former comprising the transmembrane form and the latter, constituting the active, secreted trimeric form (R. Smith & Baglioni, 1987). To emphasize the mechanistic role of renal inflammation upon blood pressure, only data from animals that also had blood pressure measurements were included in the analysis of renal proinflammatory cytokine measurements.

Table 2. Antibodies used for Western Blotting and Immunohistochemistry

Name	Function	Catalog Number	Tissues	Size	Type	Application
TNF-α	Activation of NF-KB, cell necrosis, and other pro-inflammatory cytokines (Wajant, Pfizenmaier, & Scheurich, 2003)	Santa Cruz, sc-52746	Renal cortex, renal medulla, brain, spleen, lung, liver	26 kDa (transmembrane), 51 kDa (trimer)	Mouse monoclonal	Western blotting
IL-1β	Mediates fever, induces IL-17 (Ren & Torres, 2009)	Santa Cruz, sc-7884	Renal cortex, renal medulla, spleen	17 kDa	Rabbit polyclonal	Western blotting
IL-6	Stimulates immunoglobulin production and activates T cells (Dienz & Rincon, 2009)	Santa Cruz, sc-1266	Renal cortex, renal medulla, spleen	21 kDa	Goat polyclonal	Western blotting
IL-10	Downregulates Th1 cytokine expression, MHC class II antigens (Couper, Blount, & Riley, 2008)	Santa Cruz, sc-365858	Renal cortex, renal medulla, brain, spleen, lung, liver	20 kDa	Mouse monoclonal	Western blotting
BAFF	Promotes B cell proliferation and activation (Kalled, 2005)	R&D Systems, AF2106	Renal cortex, renal medulla, spleen	21 kDa	Goat polyclonal	Western blotting
HMGB-1	Cytosolic: acts as a damage-associated molecular pattern molecule Transmembrane: In conjunction with TLR-4, activates NF-KB (Q. Chen, Guan, Zuo, Wang, & Yin, 2016)	Santa Cruz, sc-26351	Renal cortex, renal medulla, brain, spleen, lung, liver	25 kDa	Goat polyclonal	Western blotting
B-actin	Marker used to normalize protein expression	abcam, ab8227	Renal cortex, renal medulla, spleen	40 kDa	Rabbit polyclonal	Western blotting
c-Fos	Marker of neuronal activation	Synaptic Systems, 226 003	Brain	N/A	Rabbit polyclonal	immunohistochemistry
CRH	Functions in HPA axis signaling	Peninsula Laboratories, T-5007	Brain	N/A	Guinea pig polyclonal	immunohistochemistry
Anti-mouse IgG	Used to help visualize primary antibody	Vector Laboratories, BA-9200	Brain	N/A	Goat polyclonal	immunohistochemistry

ACTH and corticosterone quantification: Commercial ELISA kits were used to measure plasma ACTH and corticosterone (Enzo) three hours following LPS challenge. Plasma was collected via the retro-orbital sinus as described under "Autoantibody Quanitfication."

LPS challenge: Animals were brought up to the laboratory space at 09:30 hours and allowed to habituate for thirty minutes in their home cages. At 10:00 hours, LPS (1 mg/kg dissolved in normal saline as vehicle (Haziot et al., 1996)), or vehicle, was injected intraperitoneally at a total volume of 0.1 mL. Mice remained in the laboratory space until euthanasia at 13:00 hours. Three hours following the induction of acute inflammatory stress, anesthetized mice were transcardially perfused with 10 mL of 2% heparinized saline, then 10 mL of 4% paraformaldehyde in PBS. Brains were harvested and remained in 4% paraformaldehyde overnight. A separate subset of animals underwent a terminal blood collection followed by euthanasia.

Immunohistochemistry: After paraformaldehyde fixation, brains were kept in 30% sucrose dissolved in PBS for at least three days, until fully dehydrated. Brains were sectioned on a cryostat at 30 μm sections and kept in cryoprotectant until staining for immunohistochemistry. Brain sections were transferred to vials (1 well per vial), washed with PBS four times, and refrigerated for 30 minutes; this sequence was repeated. 100 uL H2O2 and 10 mL dH20 were mixed and distributed to tissue vials, which were then allowed to incubate at room temperature for 30 minutes. The brain slices were washed with PBS four times, then refrigerated for 30 minutes. The tissue slices were then incubated with rabbit anti-c-Fos primary antibody (SySy, Göttingen, Germany, 1:2000) dissolved in PBS diluent and allowed to incubate at 4 degrees Celsius for 2 days. Following incubation with the primary antibody, the tissue slices were washed with PBS four times, then refrigerated for 30 minutes; this sequence was repeated. Brain slices were then incubated with donkey anti-rabbit biotinylated secondary antibody (Vector Labs,

Burlingame, CA, USA; 1:10,000) diluted with PBS diluent and incubated at room temperature on a rocking platform for 2 hours. A + B Vector staining was mixed in 10 mL of PBS diluent 1 hour ahead of use. The brain slices were rinsed with PBS four times and refrigerated for thirty minutes; this sequence was repeated. The A + B Vector staining was distributed among tissue vials, which were then incubated at room temperature on a rocking platform for 1 hour. Under the fume hood, 1 DAB tablet was dissolved in 50 mL of PBS for 1 hour prior to use. The brain tissue vials were washed four times then refrigerated for thirty minutes; this sequence was repeated. The DAB solution was filtered via vacuum suction, then 2 mL of nickel ammonium solution, 1.5 mL of cobalt chloride, and 10 uL H2O2 were added. Approximately 2 mL of DAB solution was added to each tissue vial, and brain slices were timed to incubate at room temperature for 11 minutes. The brain slices were rinsed with PBS four times under the hood and then refrigerated. To prepare for incubation with a second primary antibody, the brain slices were incubated in PBS dilent at room temperature for 30 minutes, then incubated with guinea pig anti-CRF primary antibody (Peninsula, San Carlos, CA, USA; 1:1000) diluted in PBS diluent for 2 days while refrigerated. Following incubation with this second primary antibody, the brain slices were rinsed with PBS four times, then refrigerated for thirty minutes; this sequence was repeated. A goat anti-guinea pig AlexaFluor 488 secondary antibody (Fisher, Hampton, NH, USA; 1:10,000) was diluted in 10 mL of PBS diluent and distributed among the tissue vials, which incubated at room temperature on a rocking platform for 5 hours. After being rinsed with PBS for four washes, brain slices were mounted onto gel-coated slides and allowed to dry for two days. Coverslips were then affixed with Permount solution and allowed to dry for one day. Cells were imaged on an Olympus fluorescence microscope and counted manually on NIH ImageJ software.

Curcumin administration: Powdered curcumin with a purity of >94% (Sigma) was suspended in sesame oil (Sigma) and administered at a dose of 50 mg/kg animal body weight (Avasarala et al., 2013). Animals were injected intraperitoneally for 28 consecutive days with the curcumin suspension or sesame oil as vehicle in the following experimental groups (n=4-5/group): Control/Vehicle, Control/Curcumin, SLE/Vehicle, SLE/Curcumin. For a separate group of animals, curcumin or sesame oil as vehicle were intraperitoneally injected three times weekly, for 14 weeks, starting at 20 weeks of age (n = 2-3/group): Control/Vehicle, Control/Curcumin, SLE/Vehicle, SLE/Curcumin.

Unilateral cervical vagotomy: Using an aseptic surgical approach, each mouse was individually anesthetized in a rodent anesthesia chamber with 5% isoflurane mixed with oxygen. The mouse was transferred to the surgical platform and depilatory ointment was applied to remove hair from the ventral aspect of the neck, then the area was repeatedly sterilized using alternating washes of isopropyl alcohol and betadine solution. A midline incision was made from the base of the neck to the sternum, and the right cervical vagus nerve was isolated from the vascular bundle underneath the sternocleidomastoid muscle, ligated with 6-0 silk sutures, then transected (Wieczorek & Dunn, 2006). The initial incision was then sutured and the animals were allowed to recover for one hour following awakening from the anesthetized state. Animals were maintained for two weeks following vagotomy until carotid catheterization surgery, blood pressure measurements, and subsequent euthanasia.

Statistical Analysis: All statistical analyses were performed using SigmaPlot 11.2 software (Systat, Richmond, CA). Values were considered statistically different at p values < 0.05. Comparisons between two groups were made using a t-test. A two-way ANOVA (with or without repeated measures) was used in addition to the appropriate post-hoc test for comparisons between multiple groups. Based on a power analysis utilizing data from previous studies using the *NZBWF1* and *NZW* strains, an n of 6-8 per experimental group was used to achieve statistical significance for the outcome measures of mean arterial pressure, cytokine expression, autoantibody titer, glomerulosclerosis index, and albumin excretion rate.

NOTICE OF PRIOR PUBLICATION

This is to affirm that CHAPTERS I and II were previously published as the following and are hereafter being presented in their original and unchanged form:

CHAPTER I:

Lipopolysaccharide Challenge Reveals Hypothalamic-Pituitary-Adrenal Axis Dysfunction in Murine Systemic Lupus Erythematosus.

Grace S. Pham and Keisa W. Mathis.

Brain Sciences. 2018, 8, 184.

CHAPTER II:

Pharmacological potentiation of the efferent vagus nerve attenuates blood pressure and renal injury in a murine model of systemic lupus erythematosus

Grace S. Pham, Lei A. Wang, and Keisa W. Mathis

American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 2018 315:6, R1261-R1271

CHAPTER I: LIPOPOLYSACCHARIDE CHALLENGE REVEALS HYPOTHALAMIC-PITUITARY-ADRENAL AXIS DYSFUNCTION IN MURINE SYSTEMIC LUPUS ERYTHEMATOSUS[1]

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by dysautonomia in the form of decreased vagal tone and aberrant chronic inflammation (Maule et al., 1997). Due to this combination of features, the ability of the brain to regulate immune responses may be impaired in SLE. While the adaptive immune system is more commonly associated with SLE, due to the production of pathogenic autoantibodies, the innate immune system may also contribute to the pathogenesis of SLE. For instance, endogenous nucleic acids may activate toll-like receptors (Barrat et al., 2005), and antigen presentation of self-DNA complexes by neutrophils to dendritic cells may exacerbate the disease course (Lande et al., 2011). Neuroimmune interactions regulate the activity of both the innate and adaptive immune systems and their dysfunction may exacerbate autoimmune disease processes. The hypothalamic-pituitary-adrenal (HPA) axis is an endogenous neuro-endocrine-immune pathway that is commonly associated with psychological stress, but is also activated by vagus nerve afferents that are, in turn, activated by pro-inflammatory cytokines and bacterial products, such as lipopolysaccharide (LPS) (Johnston & Webster, 2009) (Figure 1). LPS directly stimulates the innate system via toll-like receptor 4 and promotes the transcription of IL-1 and TNF-α by

[1] This chapter was published as an original research article in partial fulfillment of the requirements for the degree Doctor of Philosophy (Pham & Mathis, 2018).
[2] This chapter was published as an original research article in partial fulfillment of the requirements for the degree Doctor of Philosophy (Pham et al., 2018).

immune cells (Hoshino et al., 1999). LPS (Toru Hosoi et al., 2005) and IL-1 (Goehler et al., 1998; Wieczorek & Dunn, 2006) have been shown to induce afferent vagus nerve firing that then initiates HPA axis activation, beginning with parvocellular neuronal activation in the paraventricular nuclei of the hypothalamus, which leads to corticotropin releasing factor (CRF) release. CRF induces corticotrophs in the anterior pituitary to release adrenocorticotrophic hormone (ACTH) into the bloodstream. ACTH binds to its receptors in the zona fasciculata of the adrenal cortex to prompt cortisol release (corticosterone in rodents). Cortisol inhibits both innate (Chinenov & Rogatsky, 2007) and adaptive (Elenkov, 2004) immune processes via attenuation of the inflammatory cascade (Chinenov & Rogatsky, 2007) and by shifting the inflammatory milieu from a pro-inflammatory Th1 to an anti-inflammatory Th2 profile (Elenkov, 2004), respectively. Cortisol and glucocorticoids may favor this shift to type 2-mediated anti-inflammatory responses by down-regulating pro-inflammatory Th17 cells (Bollinger, Naujoks, & Solbach, 2010). Glucocorticoids also specifically down-regulate IL-1-mediated inflammatory events by upregulating naturally-occurring IL-1 inhibitors, such as IL-1 receptor antagonist and IL-1 receptor II (Dujmovic et al., 2009). Unlike other autoimmune diseases that are characterized by prevalent upregulations of Th1 and Th17 cells, such as multiple sclerosis and rheumatoid arthritis (Raphael et al., 2015), Th1-, Th2-, and Th17-mediated events seem to cooperate in the development of lupus (Barcellini et al., 1996; Bedoya et al., 2013). Taken together and on the basis of prevalent dysautonomia and chronic peripheral inflammation, we hypothesize that HPA axis dysfunction in SLE may lead to aberrant innate immune system activity.

HPA axis responses in SLE patients and mouse models are indeed dysregulated compared to those of their healthy counterparts (Gutiérrez et al., 1998; Lechner et al., 1996; Rovenský et

al., 1998). Intuitively, this makes sense because SLE is an autoimmune disease with heightened peripheral inflammation, and deficits within the effector system that releases cortisol (or corticosterone in rodents), an anti-inflammatory steroid hormone, may contribute to chronic inflammation in SLE. Clinical studies have yielded mixed results when it comes to baseline cortisol, with some reporting decreased baseline cortisol (Straub et al., 2004) and others reporting comparable serum cortisol compared to healthy subjects (Goes et al., 2011; Gutiérrez et al., 1998). In response to insulin-induced hypoglycemia, which is expected to activate the HPA axis due to the brain sensing decreased plasma glucose, there is an attenuated plasma cortisol or corticosterone response in SLE (Gutiérrez et al., 1998; Rovenský et al., 1998). Others have shown that direct electrical stimulation of the vagal afferents, which is known to activate the HPA axis (T Hosoi et al., 2000), leads to interleukin (IL)-1β mRNA expression in the brain (T Hosoi et al., 2000) and increased plasma corticosterone (De Herdt et al., 2009), supporting this connection. On the basis of this relationship, one might expect dysfunction of the vagal afferent-HPA axis interface in SLE.

While HPA axis dysfunction is present in SLE, it is unclear whether this is due to decreased afferent vagus nerve sensitivity to inflammatory molecules (such as LPS), or whether dysfunction is present further downstream in the HPA axis, such as at the adrenal gland. We, therefore, hypothesized that female SLE mice would exhibit decreased c-Fos expression, an index of neuronal activation, in the paraventricular nucleus of the hypothalamus, as well as decreased ACTH and corticosterone responses, following LPS challenge. We additionally anticipated that basal plasma ACTH and corticosterone would be attenuated in unchallenged SLE mice compared to control strains, indicating a hypoactive HPA axis in SLE. Lastly, we expected that brain and spleen inflammation would be higher in SLE mice compared to controls,

due to inadequate HPA axis activity, so, to investigate this outcome, we measured brain and spleen pro-inflammatory cytokine expression following an LPS challenge.

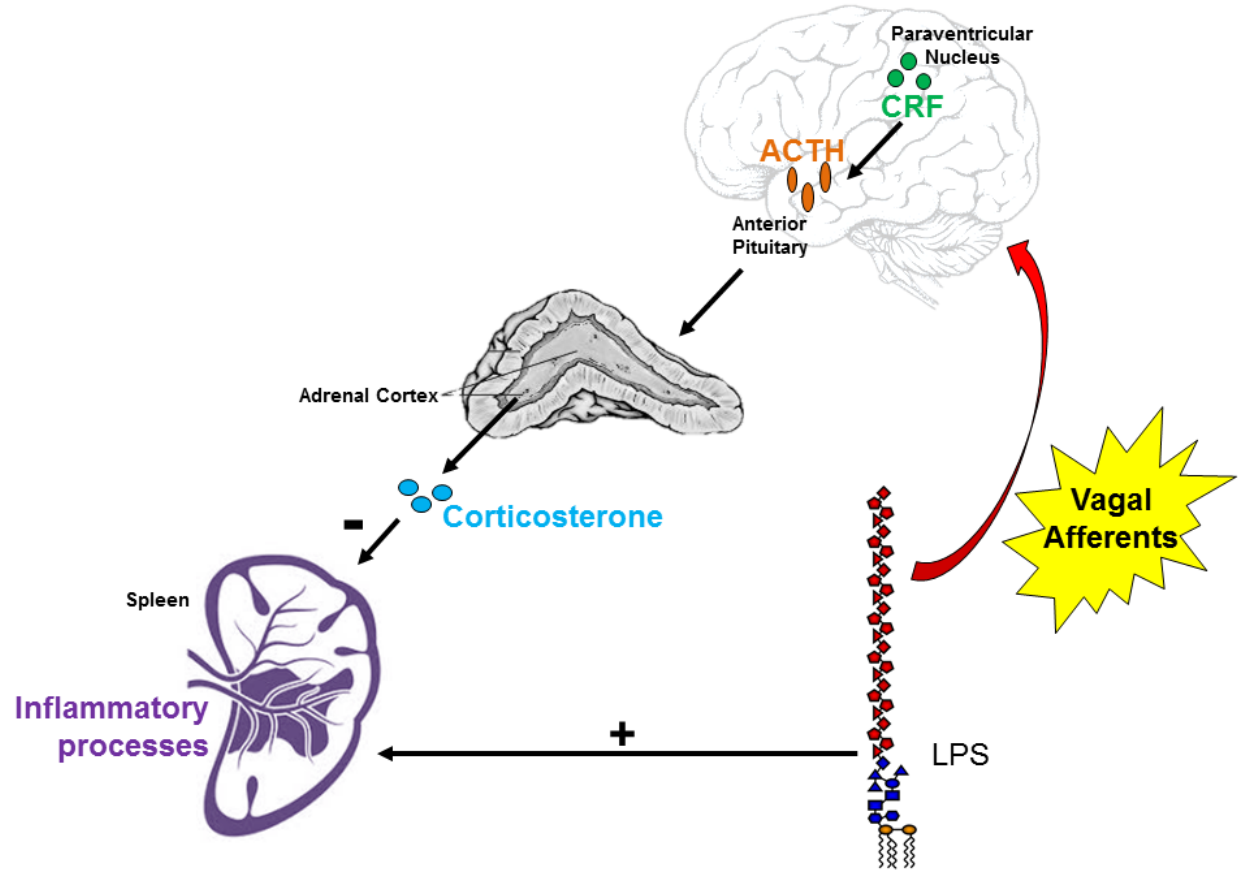


Figure 1. The vagus nerve is a conduit between the brain and immune system. Vagal afferents transmit information regarding peripheral inflammatory processes (such as lipopolysaccharide [LPS] challenge, which also induces the release of pro-inflammatory cytokines by splenic macrophages and other innate immune cells) to the nucleus tractus solitarius. This causes corticotropin releasing factor (CRF)-releasing parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus to prompt adrenocorticotrophic hormone (ACTH) release from the anterior pituitary. ACTH from the bloodstream then binds to receptors in the zona

fasciculata of the adrenal cortex to induce corticosterone release. Plasma corticosterone inhibits initiation of the inflammatory cascade.

2. Materials and Methods

Animals: Female *NZBWF1*, *NZW/LacJ,* and *C57/Bl6J* mice were obtained from Jackson Laboratories. *NZBWF1* mice, a well-established murine model of SLE (Fairley & Mathis, 2017; Mathis et al., 2013, 2012, 2011; Mathis, Wallace, et al., 2014; Venegas-Pont et al., 2011), that had an albuminuria of ≥ 300 mg/dL for two consecutive weeks were used in this study under the rationale that these mice with aggravated disease course would display the greatest amount of HPA axis dysfunction and yield larger differences compared to the *NZW* and *C57/BL6J* control strains. *NZW* mice are one of the parental strains of *NZWBF1* mice and exhibit mild autoimmunity, and were used as controls due to their similar lineage, yet lack of explicit SLE-like symptoms. *C57* mice were added as an alternate control strain as they do not share lineage with *NZBWF1* mice. All animal studies were approved by the University of North Texas Health Science Center's Institutional Animal Care and Use Committee (IACUC) and were in accordance with National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*.

Acute LPS Challenge: Animals were brought up to the laboratory space at 9:30 AM and allowed to habituate for thirty minutes. At 10 AM, LPS (1 mg/kg dissolved in normal saline as vehicle (Haziot et al., 1996)), or vehicle, was injected intraperitoneally at a total volume of 0.1 mL. Mice remained in the laboratory space until euthanasia at 1 PM. Three hours following the induction of acute inflammatory stress, anesthetized mice were transcardially perfused with 10 mL of 2%

heparinized saline, then 10 mL of 4% paraformaldehyde in PBS. Brains were harvested and remained in 4% paraformaldehyde overnight.

c-Fos and CRF Immunohistochemistry: After paraformaldehyde fixation, brains were kept in 30% sucrose dissolved in PBS for at least three days, until fully dehydrated. Brains were sectioned on a cryostat at 30 μm sections and kept in cryoprotectant until staining for immunohistochemistry as previously described (J. T. Cunningham, Grindstaff, Grindstaff, & Sullivan, 2002; J Thomas Cunningham, Mifflin, Gould, & Frazer, 2008). Brain sections were washed with PBS, then incubated with rabbit anti-c-Fos primary antibody (SySy, 1:2000) dissolved in PBS diluent. A donkey anti-rabbit biotinylated secondary antibody (Vector Labs, 1:10,000) was used along with A+B Vector staining and DAB to visualize c-Fos expression. A guinea pig anti-CRF primary antibody (Peninsula, 1:1000) was then used along with a goat anti-guinea pig AlexaFluor 488 secondary antibody (Fisher, 1:10,000) to confirm the presence and LPS-mediated activation of CRF-secreting parvocellular neurons within the paraventricular nuclei. Cells were imaged on an Olympus fluorescence microscope and counted manually on NIH ImageJ software (J Thomas Cunningham et al., 2008).

Plasma corticosterone and ACTH: A separate subset of LPS challenged SLE and control mice were anesthetized and a terminal blood sample was taken 3 hours post, in which plasma corticosterone and ACTH were later measured via commercial ELISA kits (Enzo and Phoenix Pharmaceuticals, respectively).

Brain and spleen cytokines: Brains and spleens were harvested from the subset of mice that were not used for c-Fos and CRF immunohistochemistry, then flash-frozen in liquid nitrogen and stored at -80̊ C. Tissues were weighed and homogenized with 8 times their weight of RIPA buffer plus protease inhibitors. Western blotting was performed with primary antibodies against tumor necrosis factor (TNF)-α and IL-1β (Santa Cruz, 1:250) and secondary antibodies conjugated to HRP (Rockland, 1:5000) as previously described (Fairley & Mathis, 2017). TNF-α was quantified at both the 26 and 51 kDa bands to gauge relative amounts of the transmembrane and active trimeric isoforms (R. Smith & Baglioni, 1987), respectively. Blots were imaged on a ChemiDoc imager (BioRad) and analyzed using ImageLab software (BioRad), with the bands of interest normalized to total lane protein using the stain-free quantification method, as previously reported (Fairley & Mathis, 2017).

Statistical Analysis: All data are calculated as mean ± standard error of the mean (SEM) and statistical analysis were performed using SigmaPlot 11.0 (Systat, Richmond, CA). Statistical differences ($p < 0.05$) between multiple groups were determined by two-way ANOVA followed by a Holm-Sidak post-hoc test, as specified in the accompanying figure legend. As *C57* mice were used as an alternate control strain for vehicle treatment, a t-test was used to compare unchallenged SLE and C57 groups.

RESULTS

SLE Mice had Elevated Anti-dsDNA Autoantibody Titer Compared to NZW and C57/Bl6 Mice: Plasma anti-dsDNA autoantibody titer is a commonly accepted index of disease

severity in both clinical and experimental lupus. A t-test revealed there was a significant difference in plasma dsDNA autoantibody between SLE and C57 female mice ($1.1 \times 10^5 \pm 2.9 \times 104$ vs. $5.7 \times 10^3 \pm 1.3 \times 10^3$ activity units; data not shown; n= 3 mice/group; $p = 0.036$). SLE mice also had greater plasma activity of these autoantibodies compared to NZW mice ($1.1 \times 10^5 \pm 2.9 \times 10^4$ vs. $3.9 \times 10^4 \pm 2.0 \times 10^4$, $p < 0.001$) (Figure 2). Plasma dsDNA autoantibody activity did not change 3 h post-LPS challenge in SLE or control mice.

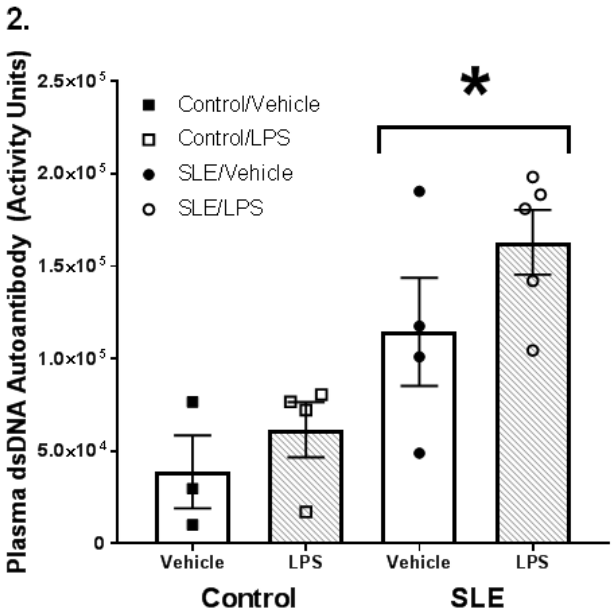
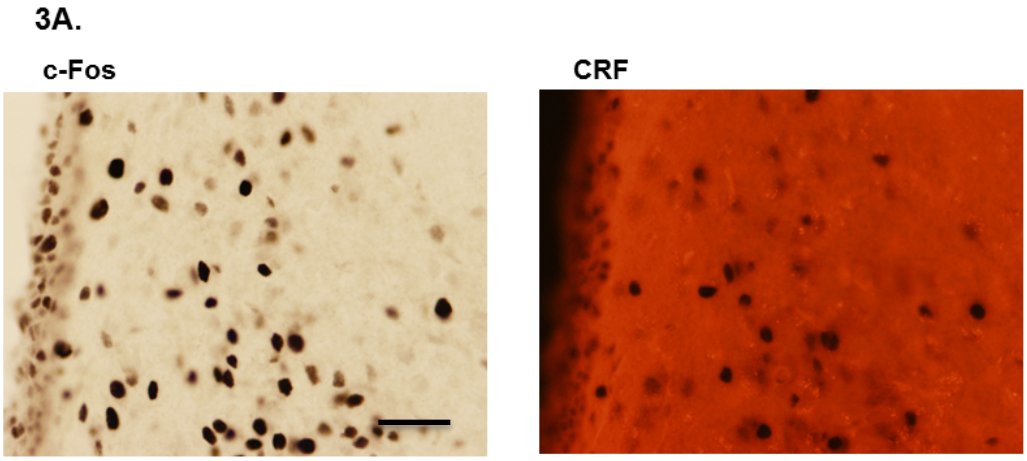


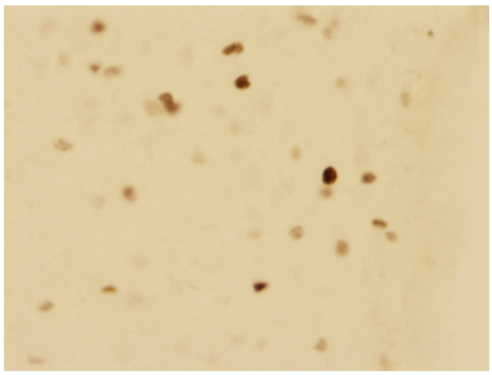
Figure 2. Systemic lupus erythematosus (SLE) mice have elevated plasma anti-dsDNA autoantibodies compared to NZW control mice, regardless of treatment group. All values are reported as mean ± SEM, and the results of the two-way ANOVA indicated a significant difference between control and SLE mice. * p SLE/Vehicle and SLE/ lipopolysaccharide (LPS) vs. Control/Vehicle.

LPS Challenge Elicits Similar Parvocellular Paraventricular Nuclei Activation between SLE and Control Mice: Following intraperitoneal injection of vehicle, paraventricular c-Fos expression did not differ between SLE and NZW mice (17.0 ± 3.2 vs. 14.2 ± 2.9 cells/field; data not shown; p = 0.537). Similarly, there were no differences in c-Fos expression between SLE and control (NZW) mice (17.0 ± 3.2 vs. 18.2 ± 1.9 cells/field, p = 0.867). LPS increased c-Fos neuronal expression in both SLE and control mice compared with their vehicle-treated counterparts (39.2 ± 5.5 vs. 17.0 ± 3.2 cells/field, p = 0.007 for SLE; 42.3 ± 7.8 vs. 18.2 ± 1.9 cells/field, p = 0.004 for controls) (Figure 3C), and by the same magnitude (39.2 ± 5.5 vs. 42.3 ± 7.8 cells/field, p = 0.672).

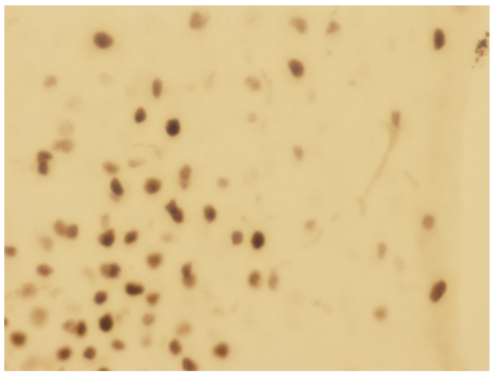


3B.

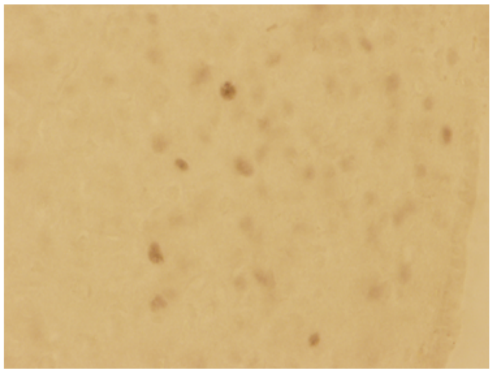
Control/Vehicle



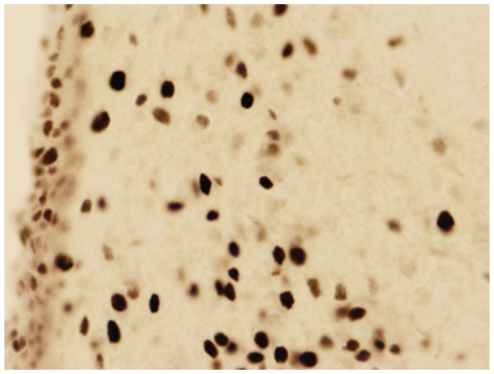
Control/LPS



SLE/Vehicle



SLE/LPS



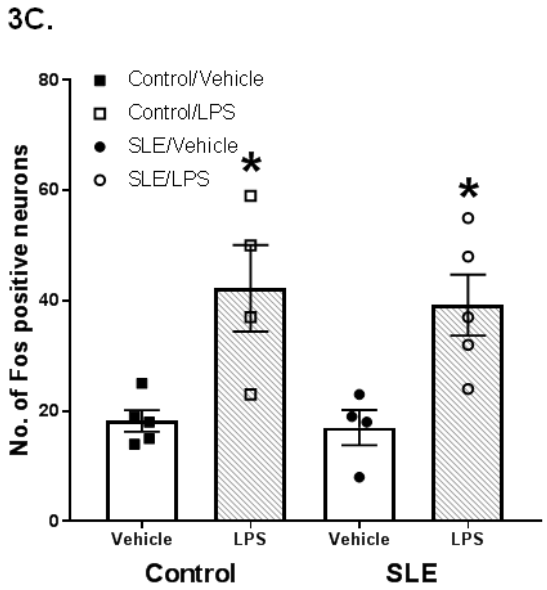
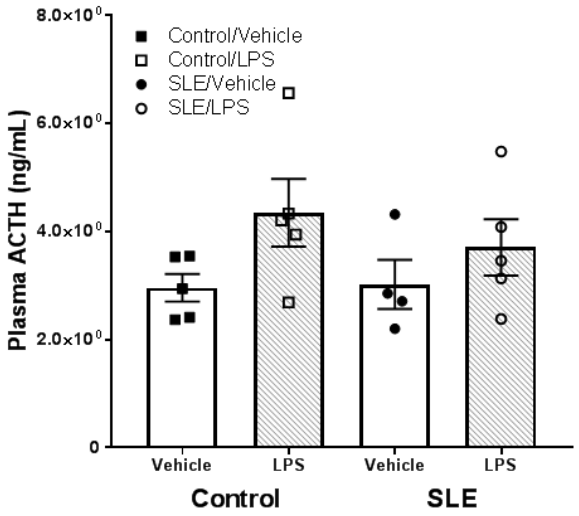


Figure 3. (A) Examples of c-Fos and corticotropin-releasing factor (CRF) staining in the paraventricular nucleus (PVN) parvocellularis. As vasopressin-secreting neurons also comprise the parvocellular region of the PVN, CRF staining was used to verify the presence and activation of CRF-secreting parvocellular neurons in the PVN. (B) Representative images of the paraventricular nucleus taken at 40× from systemic lupus erythematosus (SLE) and control mice. (C) Quantitative representation of positive cell counts between lipopolysaccharide (LPS)-treated SLE and control mice. All values are reported as mean ± SEM, and the results of the two-way ANOVA indicated significant differences between LPS-challenged SLE and control mice compared to their vehicle-treated counterparts. * p SLE/LPS vs. SLE/Vehicle and p Control/LPS vs. Control/Vehicle. Scale bar denotes 2μm.

LPS has Differential Effects on HPA Axis Hormones in SLE Mice: Following vehicle injection, plasma ACTH did not differ between SLE and C57 mice (3.0 ± 0.5 vs. 3.7 ± 0.4 ng/mL; p = 0.378, data not shown), nor SLE and control (NZW) mice (3.0 ± 0.5 vs. 3.0 ± 0.3

ng/mL; p = 0.913; Figure 4A). Plasma ACTH also did not change following LPS challenge in both SLE and control mice (3.0 ± 0.5 vs. 3.7 ± 0.5 and 3.0 ± 0.3 vs. 4.3 ± 0.6 ng/mL, p = 0.393, respectively).

4A.



4B.

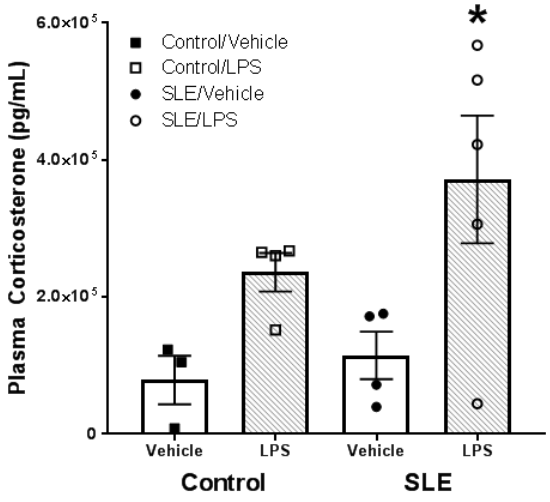
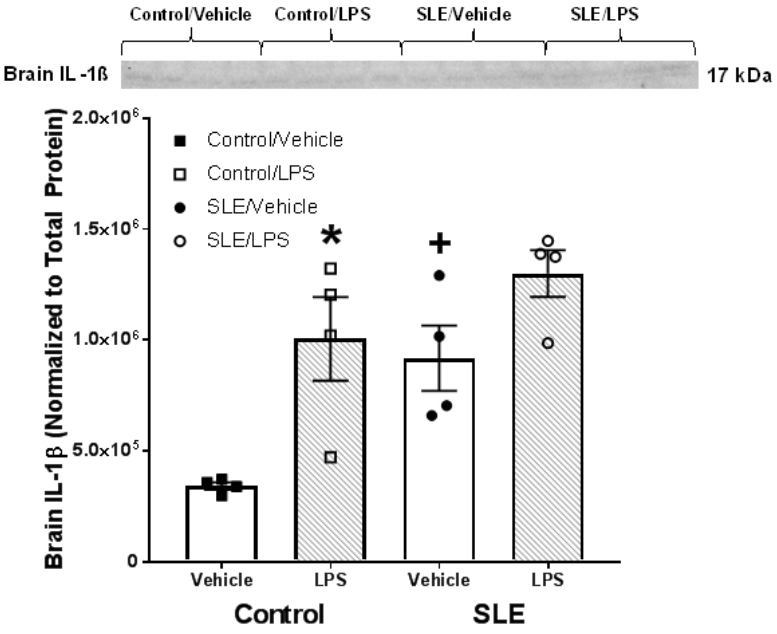


Figure 4. (A) Lipopolysaccharide (LPS) challenge does not affect plasma adrenocorticotrophic hormone (ACTH) concentration in systemic lupus erythematosus (SLE) and control mice. (B) LPS challenge provokes a significant increase in plasma corticosterone in SLE mice. All values are reported as mean ± SEM, and the results of the two-way ANOVA indicated a significant difference between LPS-challenged SLE mice and their n counterparts. * p SLE/LPS vs. SLE/Vehicle.

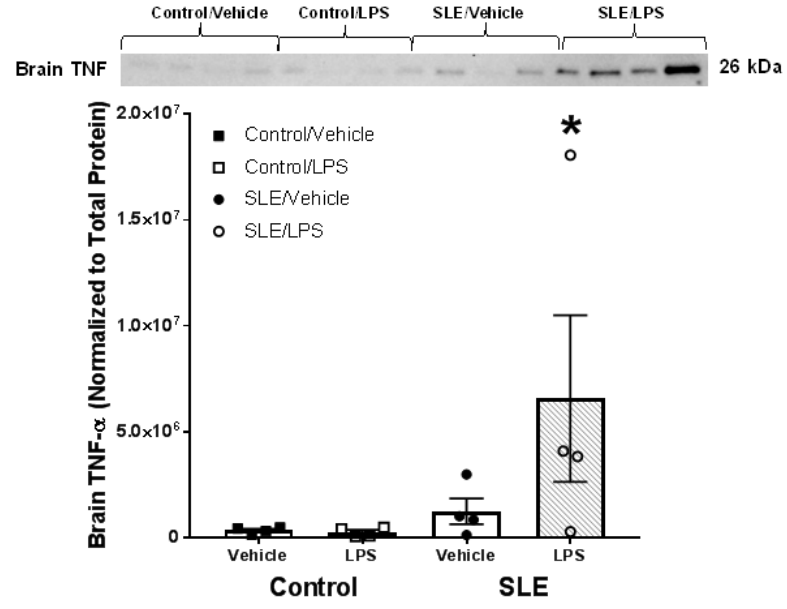
Plasma corticosterone was not different in SLE mice compared to C57 mice ($1.1 \times 10^5 \pm 3.5 \times 10^4$ vs. $4.7 \times 10^4 \pm 2.0 \times 10^4$; p = 0.348, data not shown). Similarly, plasma corticosterone was not different in SLE mice compared to NZW controls ($1.1 \times 10^5 \pm 3.5 \times 10^4$ vs. $7.9 \times 10^4 \pm 3.6 \times 10^4$; p = 0.613; Figure 4B). LPS-challenged SLE mice had increased plasma corticosterone compared to vehicle-treated SLE mice ($3.7 \times 10^5 \pm 9.3 \times 10^4$ vs. $1.1 \times 10^5 \pm 3.5 \times 10^4$; p = 0.009). By contrast, plasma corticosterone was not significantly altered in LPS-challenged control mice compared to their vehicle-treated counterparts ($2.4 \times 10^5 \pm 2.8 \times 10^4$ vs. $7.9 \times 10^4 \pm 3.6 \times 10^4$; p= 0.152; Figure 4B).

LPS Alters Brain and Splenic Pro-Inflammatory Cytokine Expression: Brain IL-1β was elevated in SLE mice compared to controls ($9.2 \times 10^5 \pm 1.5 \times 105$vs. $3.4 \times 10^5 \pm 1.6 \times 10^4$, p = 0.009) (Figure 5A). LPS-challenged control mice had elevated brain IL-1β compared to vehicle-treated controls ($1.0 \times 10^6 \pm 1.9 \times 10^5$ vs. $3.4 \times 10^5 \pm 1.6 \times 10^4$; p = 0.004). However, LPS challenge did not significantly alter brain IL-1β in SLE mice, although there was a higher trend than vehicle-treated SLE mice. ($1.3 \times 10^6 \pm 1.1 \times 10^5$ vs. $9.2 \times 10^5 \pm 1.5 \times 10^5$; p = 0.062).

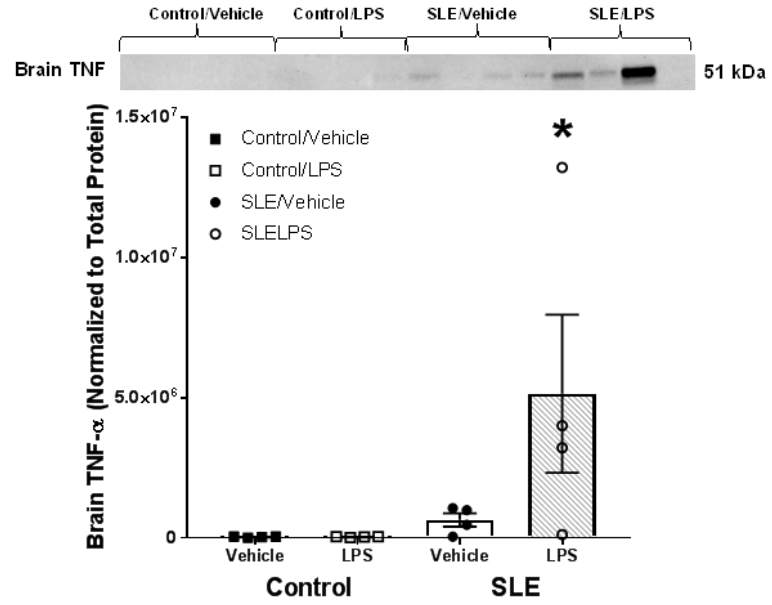
5A.


Control/Vehicle
Control/LPS
SLE/Vehicle
SLE/LPS
Brain IL-1ß
17 kDa
Brain IL-1β (Normalized to Total Protein)
2.0x10^6
1.5x10^6
1.0x10^6
5.0x10^5
0
Control/Vehicle
Control/LPS
SLE/Vehicle
SLE/LPS
*
+
Vehicle
LPS
Vehicle
LPS
Control
SLE

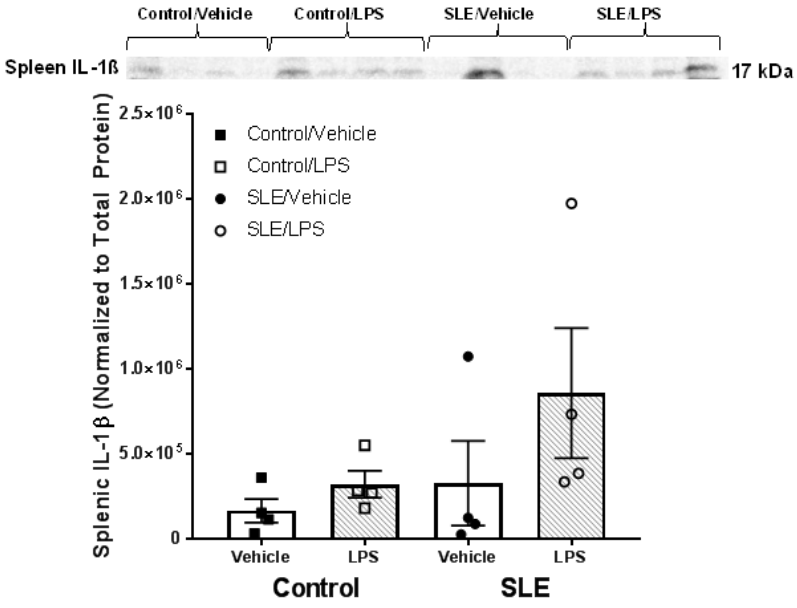
5B.


Control/Vehicle
Control/LPS
SLE/Vehicle
SLE/LPS
Brain TNF
26 kDa
Brain TNF-α (Normalized to Total Protein)
2.0x10^7
1.5x10^7
1.0x10^7
5.0x10^6
0
Control/Vehicle
Control/LPS
SLE/Vehicle
SLE/LPS
*
Vehicle
LPS
Vehicle
LPS
Control
SLE

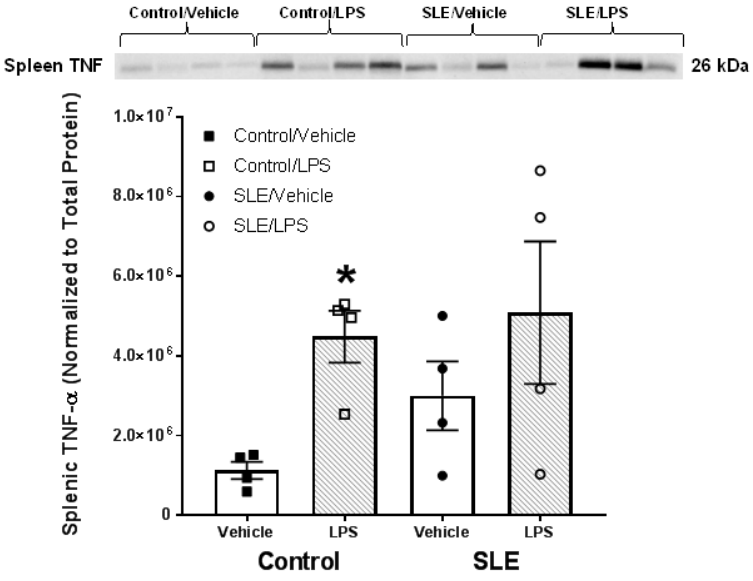
5C.


Control/Vehicle
Control/LPS
SLE/Vehicle
SLE/LPS
Brain TNF
51 kDa
Brain TNF-α (Normalized to Total Protein)
1.5x10^7
1.0x10^7
5.0x10^6
0
Control/Vehicle
Control/LPS
SLE/Vehicle
SLELPS
*
Vehicle
LPS
Vehicle
LPS
Control
SLE

5D.


Control/Vehicle
Control/LPS
SLE/Vehicle
SLE/LPS
Spleen IL-1ß
17 kDa
Splenic IL-1β (Normalized to Total Protein)
2.5x10^6
2.0x10^6
1.5x10^6
1.0x10^6
5.0x10^5
0
Control/Vehicle
Control/LPS
SLE/Vehicle
SLE/LPS
Vehicle
LPS
Vehicle
LPS
Control
SLE

5E.


Control/Vehicle
Control/LPS
SLE/Vehicle
SLE/LPS
Spleen TNF
26 kDa
Splenic TNF-α (Normalized to Total Protein)
1.0×10^7
8.0×10^6
6.0×10^6
4.0×10^6
2.0×10^6
0
Control/Vehicle
Control/LPS
SLE/Vehicle
SLE/LPS
*
Vehicle
LPS
Vehicle
LPS
Control
SLE

5F.

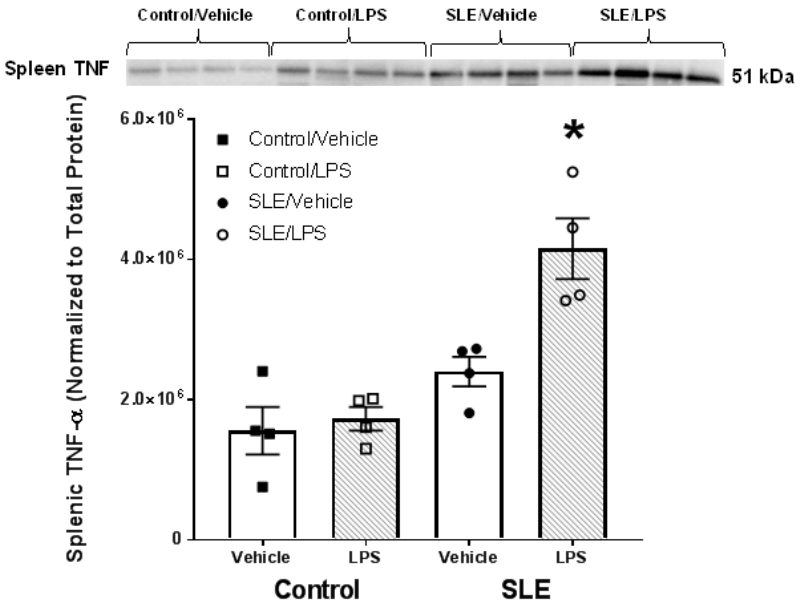

Control/Vehicle
Control/LPS
SLE/Vehicle
SLE/LPS
Spleen TNF
51 kDa
Splenic TNF-α (Normalized to Total Protein)
6.0×10^6
4.0×10^6
2.0×10^6
0
Control/Vehicle
Control/LPS
SLE/Vehicle
SLE/LPS
*
Vehicle
LPS
Vehicle
LPS
Control
SLE

Figure 5. Pro-inflammatory cytokine expression was measured in brains and spleens of lipopolysaccharide (LPS)-challenged and vehicle-treated systemic lupus erythematosus (SLE) and control (NZW) mice. (A) Brain IL-1β was elevated in SLE mice compared to controls. LPS-challenged controls had elevated brain IL-1β compared to their vehicle-treated counterparts. (B, C) Brain TNF-α (both 26 and 51 kDa) did not differ between SLE and control mice. LPS-challenged SLE mice had higher TNF-α than vehicle-treated SLE mice. This elevation was not seen in control mice. (D) Splenic IL-1β did not significantly differ between any groups of mice. (E, F) Splenic TNF-α did not differ between SLE and control mice. The 26 kDa isoform of splenic TNF-α was elevated following LPS challenge in control mice when compared their vehicle-treated counterparts, but this was not observed in SLE mice. Contrastingly, the 51 kDa isoform of splenic TNF-α was increased in LPS-challenged SLE mice compared to their vehicle-treated counterparts, and this phenomenon was not seen in control mice. All values are reported as mean ± SEM, and results from a two-way ANOVA indicate significant differences. * p SLE/LPS vs. SLE/Vehicle and Control/LPS vs. Control/Vehicle; + p SLE/Vehicle vs. Control/Vehicle.

Brain TNF-α (both 26 and 51 kDa isoforms) did not differ between SLE and control mice ($1.2 \times 10^6 \pm 6.1 \times 10^5$ vs. $3.4 \times 10^5 \pm 7.6 \times 104$ intensity units, $p = 0.754$ for 26 kDa; $6.4 \times 10^5 \pm 2.4 \times 10^5$ vs. $3.3 \times 10^4 \pm 7.9 \times 103$ intensity units, $p = 0.766$ for 51 kDa) (Figure 5B,C). LPS-challenged SLE mice had higher 51 kDa brain TNF-α expression ($5.1 \times 10^6 \pm 2.8 \times 10^6$ vs. $6.4 \times 10^5 \pm 2.4 \times 10^5$ intensity units, $p = 0.044$ for 51 kDa), but not 26 kDa ($6.6 \times 10^6 \pm 3.9 \times 10^6$ vs. $1.2 \times 10^6 \pm 6.1 \times 10^5$ intensity units, $p = 0.083$ for 26 kDa), compared to their vehicle-treated counterparts. Curiously, LPS challenge did not result in differences in brain TNF-α in control mice ($2.6 \times 10^5 \pm 1.1 \times 10^5$ vs. $3.4 \times 10^5 \pm 7.6 \times 10^4$ intensity units, $p = 0.978$ for 26 kDa; $1.1 \times$

$10^5 \pm 2.4 \times 10^4$ vs. $3.3 \times 10^4 \pm 7.9 \times 103$ intensity units, p = 0.968 for 51 kDa). Lastly, LPS-challenged SLE mice had increased brain TNF-α compared to LPS-challenged control mice ($6.6 \times 10^6 \pm 3.9 \times 10^6$ vs. $2.6 \times 10^5 \pm 1.1 \times 10^5$ intensity units, p = 0.045 for 26 kDa; $5.1 \times 10^6 \pm 2.8 \times 10^6$ vs. $1.1 \times 10^5 \pm 2.4 \times 10^4$ intensity units, p = 0.027 for 51 kDa).

Splenic IL-1β did not differ between SLE and control mice ($3.3 \times 10^5 \pm 2.5 \times 10^5$ vs. $1.7 \times 10^5 \pm 7.0 \times 10^4$ intensity units, p = 0.633) (Figure 5D). LPS challenge did not affect splenic IL-1β in SLE ($8.6 \times 10^5 \pm 3.8 \times 10^5$ vs. $3.3 \times 10^5 \pm 2.5 \times 10^5$ intensity units, p = 0.136) nor control ($3.2 \times 10^5 \pm 8.0 \times 10^4$ vs. $1.7 \times 10^5 \pm 7.0 \times 10^4$ intensity units, p = 0.646) mice. Splenic TNF-α (26 and 51 kDa) did not differ between vehicle-treated SLE and control mice ($3.0 \times 10^6 \pm 8.6 \times 10^5$ vs. $1.1 \times 10^6 \pm 2.2 \times 10^5$ intensity units, p = 0.231 for 26 kDa; $2.4 \times 10^6 \pm 2.1 \times 10^5$ vs. $1.6 \times 10^6 \pm 3.4 \times 105$ intensity units, p = 0.076 for 51 kDa) (Figure 5E,F). The 26 kDa form of splenic TNF-α was not significantly elevated in LPS-challenged SLE mice compared to their counterparts ($5.1 \times 10^6 \pm 1.8 \times 10^5$ vs. $3.0 \times 10^6 \pm 8.6 \times 10^5$ intensity units, p = 0.187), however, was increased in LPS-challenged controls compared to vehicle-treated controls ($4.5 \times 10^6 \pm 6.5 \times 10^5$ vs. $1.1 \times 10^6 \pm 2.2 \times 10^5$ intensity units, p = 0.043). Contrastingly, the 51 kDa form of splenic TNF-α was elevated in SLE mice compared to their vehicle-treated counterparts following LPS challenge ($4.2 \times 10^6 \pm 4.3 \times 10^5$ vs. $2.4 \times 10^6 \pm 2.1 \times 10^5$ intensity units, p = 0.002), while there was no change between LPS-challenged and vehicle-treated controls ($1.7 \times 10^6 \pm 1.7 \times 10^5$ vs. $1.6 \times 10^6 \pm 3.4 \times 10^5$ intensity units, p = 0.705).

DISCUSSION

The HPA axis is intriguing in conditions of chronic inflammation because, despite its known powerful ability to counteract inflammation, this neuro-endocrine-immune pathway is not always effective. This is the case in the chronic autoimmune inflammatory disorder SLE, and it is unknown whether the dysfunction of the HPA axis occurs at the level of the afferent vagal-hypothalamic PVN interface, the anterior pituitary, or the adrenals. We examined this important question by monitoring innate immune responses in the brain and spleen following an acute inflammatory stimulus (i.e., LPS injection). We found that, following an acute LPS challenge, (1) c-Fos expression in the PVN was similar between SLE and control mice, (2) plasma ACTH was not altered in SLE or control mice, (3) SLE mice had an increased plasma corticosterone whereas controls did not, and (4) brain and spleen TNF-α was increased in SLE mice compared to their vehicle-treated counterparts.

SLE remains a major health disparity and disease burden worldwide. Recent epidemiological studies estimate the prevalence of SLE to be 6 to 178 cases per 100,000 worldwide (Pons-Estel, Alarcón, Scofield, Reinlib, & Cooper, 2010). While the exact etiology of SLE is unknown, current research supports the interaction between genetic and environmental factors that culminates in a loss of self-tolerance and over-activation of the adaptive immune, specifically T cells and B cells (G. S. Cooper et al., 1998). All organ systems may be damaged in SLE and this is typically the result of inflammatory immune complexes formed from pathogenic autoantibodies (anti-dsDNA being the most diagnostic and prognostic) because of immune cell over-activation (Ippolito et al., 2011; Wajed, Ahmad, Durrington, & Bruce, 2004). As chronic inflammation mediates end-organ damage, treatment is focused upon preventing disease flares and suppressing the immune system, which is mainly achieved through exogenous

glucocorticoids and immunomodulatory agents that suppress specific immune cell populations or immune processes (Amissah-Arthur & Gordon, 2010).

A large body of research investigating HPA axis dysfunction in SLE and other autoimmune diseases had taken place during the late 1990s and early 2000s, but inquiry within this niche has since dwindled. It is difficult to elucidate whether HPA axis dysfunction occurs before the onset of rheumatic diseases, but the chronic inflammatory burden in autoimmune diseases does impair the HPA axis (Crofford, 2002). The exact etiology for HPA axis dysfunction in SLE is not known, but may be the result of antibody-mediated neuroinflammation, as others have found that autoantibodies mediate neuropsychiatric disease presentation in the NZM88 murine model of SLE (Mondal, Saha, Miller, Seegal, & Lawrence, 2008). Contemporary biomedical research in autoimmune diseases is more focused towards immunosuppressive and immunomodulatory agents that target specific immune cell populations or proteins implicated in disease maintenance. There are multiple challenges in elucidating disease mechanisms involving the HPA in autoimmune diseases: (a) both human patients and experimental animal models present with highly variable disease phenotypes, (b) human patients are likely to be on exogenous corticosteroids or immunomodulatory drugs, and (c) the time courses for both HPA axis development and SLE disease progression may vary with other external factors, such as timing of maternal separation (Catallani, Palma, Gil, & Suchecki, 2008). However, as a major endogenous regulator of systemic inflammation, the HPA axis and its characteristic dysfunction in autoimmune diseases are worth thorough comprehension. We utilized LPS as a means of gauging HPA axis dysfunction because we were specifically interested in the role of vagal afferents in transmitting information about peripheral inflammation centrally, and others have established that LPS activates the HPA axis through vagal afferents

(Beishuizen & Thijs, 2003). Insulin-induced hypoglycemic challenge has also been used to investigate HPA axis dysfunction in murine SLE (Gutiérrez et al., 1998; Rovenský et al., 1998). However, because the *NZBWF1* strain presents with features of metabolic syndrome and we did not want this characteristic to also affect plasma corticosterone and because our interest remains with that of the vagus nerve's role in HPA axis dysfunction, we used LPS in this study (Ryan, McLemore, & Hendrix, 2006).

The finding that c-Fos expression in the hypothalamic parvocellular neurons was comparable between SLE mice and control strains indicates that the afferent vagal nerve-PVN interaction may not be compromised in SLE (Figure 3C). Likewise, no differences in plasma ACTH were observed between SLE and control mice (Figure 4A). Clinical studies have been mixed as to whether basal serum concentrations of cortisol, an anti-inflammatory steroid hormone, are lower in patients with SLE (Straub et al., 2004). In our study we did not observe decreased plasma corticosterone in our unchallenged SLE mice compared to control strains (Figure 4B). Other animal studies support these data by demonstrating a diminished ability to raise plasma corticosterone compared to controls following insulin-induced hypoglycemia (Lechner et al., 1996). Conversely, in the current study, we found there was a greater increase in plasma corticosterone in SLE mice compared to controls following a challenge with a different stressor. The dose chosen for LPS challenge (1 mg/kg body weight (Haziot et al., 1996)) was not sufficient to elevate plasma corticosterone in control mice relative to vehicle-treated controls, nor plasma ACTH in both strains of mice (Figure 4A). However, SLE mice that had received LPS had elevated plasma corticosterone compared to vehicle-treated SLE mice (Figure 4B). This finding was contrary to our anticipated outcome, that the same dosage of LPS would result in a lesser increase of plasma corticosterone in SLE mice compared to controls. This could be due to

different processing of inflammatory stimuli in experimental SLE compared to wild-type mice, especially in light of chronically elevated peripheral inflammation in SLE. Additionally, an in vitro study by Klein et al. demonstrated that lymphocytes isolated from SLE patients catabolize cortisol at a decreased rate compared to lymphocytes from healthy subjects (Klein, A. Buskila, D. Gladman, D. Bruser, B. Malkin, 1990). Perhaps this may also be the case concerning lymphocytes and adaptive immune cells in SLE mice, and may help explain the increased plasma corticosterone in SLE mice compared to controls.

Animal studies using wild-type rats have demonstrated that plasma TNF-α decreases significantly following LPS administration and within 2 h of peak plasma corticosterone concentration, so we expected similar responses in our similarly timed study (Givalois et al., 1994). However, both brain and spleen pro-inflammatory cytokines were elevated in SLE mice that had received LPS (Figure 5A–D) despite increased plasma corticosterone. The heightened peripheral and central inflammation, as well as the increase in plasma corticosterone provoked by LPS, suggest that the systemic inflammatory response, as well as the HPA axis response, may be more sensitive and easily exacerbated in SLE. Perhaps the magnitude of the corticosterone increase elicited by LPS challenge was not sufficient to decrease peripheral inflammation in SLE mice. Alternatively, it may be that glucocorticoid receptor desensitization occurs more frequently in SLE. This latter phenomenon makes sense in light of SLE patients that experience glucocorticoid receptor desensitization as a result of exogenous glucocorticoid therapy. Additionally, glucocorticoid receptor polymorphisms have also been associated with SLE in certain populations, which suggests that glucocorticoid receptor desensitization could be a feature of SLE (Y.-F. Chen et al., 2017; Zou et al., 2013). As in SLE patients, endogenous mechanisms of resistance to glucocorticoids operate in SLE mice and the augmented production

of endogenous corticosterone in response to LPS is a compensatory, though ineffective, attempt (Gao et al., 2018). Interestingly, resistance to both endogenous and exogenous glucocorticoids is primarily induced by the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) that plays a role in animal models of autoimmune disease, such as multiple sclerosis, Guillain-Barre syndrome, and SLE (Fagone et al., 2018; Lapter et al., 2011; Nicoletti et al., 2005). It is of particular relevance in the context of this paper that *NZBWF1* SLE-prone mice have elevated expression of molecules of the CD74/MIF pathway on B cells and in two target organs, namely, brain hippocampi and kidneys of SLE-afflicted mice, which may also contribute to corticosteroid resistance in SLE (Lapter et al., 2011).

Exogenous glucocorticoids emerged in the 1950s, and have remained a mainstay in SLE treatment (Bollet, 1955). However, synthetic glucocorticoids have multiple unwanted side effects, most commonly, weight gain, but also severe side effects, like compression fractures and psychosis (Kasturi & Sammaritano, 2016). Prior to the introduction of synthetic glucocorticoids, such as prednisone, ACTH and cortisone were the standard of treatment (Harris-Jones, 1956). Recently, ACTH-containing gels and supplements have re-emerged as an alternative for patients who cannot tolerate glucocorticoids (X. Li et al., 2016). As an active agent, ACTH exerts direct anti-inflammatory effects directly through melanocortin (M)-1-5 receptors, which leads to nuclear factor kappa B inhibition, as well as indirect effects via stimulation of cortisol release (Fiechtner & Montroy, 2014). While contemporary pharmaceuticals have shifted towards B cell depletion and other B cell-directed modalities, the HPA axis continues to be relevant in the treatment of severe SLE and synthetic hormones targeting the HPA axis have greatly decreased morbidity and mortality since their widespread use (Lightstone et al., 2018). For these reasons,

studies like ours help to clearly define nuances in HPA axis dysfunction, which could assist with more effective drug regimens.

Vagus nerve stimulation (VNS) has also been indicated for autoimmune diseases, as it activates the cholinergic anti-inflammatory pathway (Bonaz & Pellissier, 2016; L. V. Borovikova, Ivanova, Zhang, et al., 2000a; Inoue et al., 2016a; F. a Koopman, Schuurman, & Vervoordeldonk, 2014). However, others have shown that electrical vagus nerve stimulation can activate afferent vagal fibers and directly prompt HPA axis activity (T Hosoi et al., 2000). Since 80% of vagal nerve fibers are afferent, and it is difficult to selectively stimulate afferent or efferent fibers, we posit that electrical vagus nerve stimulators will lead to afferent vagal fiber conduction and increased HPA axis activity, although current studies and clinical trials have not focused on validating the role of the HPA axis in VNS's therapeutic effects.

In summary, our findings that LPS challenge increases plasma corticosterone in SLE mice, yet results in greater brain and spleen inflammation, appear to confirm our hypothesis that, in SLE, HPA axis activity may not be adequate to combat inflammation. The existing research into HPA axis dysfunction in SLE and other autoimmune diseases is fraught with variation stemming from clinical populations, experimental animal models, medication status, and disease flares. Our current studies have successfully brought attention to the phenomenon of HPA axis dysfunction at the level of effector glands and hormones, and future studies should focus on enhanced sensitivity of corticosterone release and corticosterone receptor sensitization, and the effect this has in chronic inflammatory conditions like SLE.

SUMMARY

The HPA axis is an endogenous neuro-immune mechanism that responds to stress and modulates inflammation. While chronic dysfunction of the HPA axis has been identified in disease models including depression and stress-induced hypertension, less is known about the role of the HPA axis in lupus hypertension, much less essential hypertension. This study was focused on the HPA axis and possible dysfunction within several components of this neuro-immune mechanism in the setting of murine SLE. The HPA axis has been investigated in both clinical and preclinical SLE studies, without thorough characterization, although it is widely agreed that SLE patients may be susceptible to HPA axis dysfunction due to exogenous corticosteroids comprising a mainstay of treatment. The main conclusions gleaned from this chapter are that (1) vagal afferent sensitivity between SLE and disease-free animals may not differ substantially, (2) LPS challenge elicits a greater peripheral cytokine response in SLE mice, and (3) plasma corticosterone release in response to LPS challenge may not be appropriate to address the endotoxin-induced inflammatory cytokine response in SLE mice. Taken together, these findings suggest that adrenal gland dysfunction may be present in SLE and that responses to infection may likewise be abnormal in SLE patients, who have been documented to succumb to infectious diseases to a greater extent than their healthy counterparts (Gladman, Hussain, Iban, & Urowitz, 2002).

CHAPTER II: PHARMACOLOGICAL POTENTIATION OF THE EFFERENT VAGUS NERVE ATTENUATES BLOOD PRESSURE AND RENAL INJURY IN A MURINE MODEL OF SYSTEMIC LUPUS ERYTHEMATOSUS[2]

INTRODUCTION

Hypertension affects nearly one-half of the adult population in the United States, and this figure will only increase in the coming decades (Whelton et al., 2018). Despite growing awareness of the numerous etiologies of hypertension, at least 15–20% of hypertensive patients are resistant to current treatment modalities (Pimenta & Calhoun, 2012). It is, therefore, necessary to investigate novel mechanisms involved, to develop new therapeutic strategies to target the disease.

Recent studies support the role of dysregulated immune cell activity and chronic inflammation in the development and maintenance of hypertension. In general, elevated circulating proinflammatory cytokines [i.e., TNF-α (5), IL-6 (5), and B cell activating factor (BAFF) (Chan et al., 2015), among others] are correlated with hypertension, indicating that peripheral inflammation may mechanistically contribute to chronic increases in blood pressure (Blasi et al., 2003; Chan et al., 2015; Margretardottir et al., 2009). Renal inflammation, in particular, also directly affects kidney processes that may promote increases in blood pressure. TNF-α in the kidney, for example, has been shown to increase renal vascular resistance (Shahid, Francis, & Majid, 2008), as well as, prompt immune cell-mediated renal damage (Ramseyer & Garvin, 2013; Zhang et al., 2014). Blockade of TNF-α, in turn, attenuates both renal injury and

[2] This chapter was published as an original research article in partial fulfillment of the requirements for the degree Doctor of Philosophy (Pham et al., 2018).

hypertension in a variety of experimental animal models (Elmarakby, Quigley, Imig, Pollock, & Pollock, 2008; Elmarakby, Quigley, Pollock, & Imig, 2006; Tran, MacLeod, & McNeill, 2009; Venegas-Pont et al., 2010). In response, the finding that immunomodulatory drugs are therapeutic in both hypertensive animal models (Taylor & Ryan, 2017) and in human patients with underlying chronic inflammation (Ginzler et al., 2005) warrants further investigation into the mechanisms that regulate renal inflammation, in particular, due to the role of the kidneys in the long-term control of blood pressure.

Endogenous nervous system/immune system interactions are known to control inflammation both acutely (Ohki et al., 2017) and chronically (Deleo, Tanga, & Tawfik, 2004; Kane, 2005). Tracey and colleagues have demonstrated that the vagally mediated cholinergic anti-inflammatory pathway is capable of attenuating splenic inflammation on an immediate basis, in the context of an acute inflammatory stressor (Andersson & Tracey, 2012; Bernik et al., 2002; L. V. Borovikova, Ivanova, Zhang, et al., 2000a; Ji et al., 2014). However, the role of this nerve-to-spleen pathway in chronic, or even inherent inflammatory states, in the case of autoimmunity, is not as well delineated. We have identified the autoimmune inflammatory disease systemic lupus erythematosus (SLE) as an appropriate model to investigate neuroimmune mechanisms that regulate chronic inflammation, which, if left unchecked, can lead to hypertension (Mathis, 2015; J. M. Sabio et al., 2011). Indeed, the prevalence of hypertension in SLE is exceedingly high, especially in its target demographic of reproductive-age women who are normally protected from hypertension and cardiovascular disease (J. M. Sabio et al., 2011). Dysautonomia is also associated with SLE, and these patients have decreased heart rate variability in the high-frequency power domain, which indicates impaired efferent vagal tone (Thanou et al., 2016). A functioning efferent vagus nerve is thought to be necessary for proper

neuroimmune communication in the cholinergic anti-inflammatory pathway, and since efferent vagal tone is decreased in SLE, perhaps the efficacy of this pathway in reducing inflammation is compromised.

Galantamine is a reversible acetylcholinesterase inhibitor capable of crossing the blood-brain barrier and can act upon M1 muscarinic receptors centrally to potentiate efferent vagus nerve activity (Pavlov et al., 2009). In the present study, we hypothesized that pharmacological stimulation of the efferent vagus nerve, using galantamine, would enhance the cholinergic anti-inflammatory pathway and attenuate systemic inflammation, thus protecting from renal inflammation, renal injury, and hypertension in a mouse model of SLE.

MATERIALS AND METHODS

Animals: Female SLE (*NZBWF1)* and control (*NZW/LacJ*) mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 5-6 weeks of age. All mice were maintained on a 12-hour light/dark cycle in temperature-controlled rooms with access to food and water *ad libitum.* Mice were monitored starting at 29 weeks of age, an age at which SLE mice have already developed autoantibodies and renal disease. All animal studies were approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee (IACUC) and were in accordance with National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*.

Acute Vagus Nerve Recording: Vagus nerve recordings were performed on anesthetized animals. SLE and control mice were weighed and intraperitoneally injected with alpha-

chloralose (80 mg/kg) and urethane (1000 mg/kg). Fur was shaved off the cervical region and mice were instrumented with a Millar catheter (Model SPR 671, Millar) in the left carotid artery to continuously record blood pressure and heart rate. The right cervical vagus nerve was localized and placed on a hooked platinum-iridium electrode (36 G). The electrode was encased in QuikSil silicone gel to minimize noise and interference from the environment. Electric signals were amplified (x 200,000) and filtered (band pass: 10-10,000 Hz) to represent vagus nerve activity. A 10-minute baseline was recorded in Spike2 (Cambridge Electronic Design) and then a single dose of galantamine (4 mg/kg dissolved in saline, IP) was injected, with the following vagus nerve activity recorded. Vagal activity was allowed to recover to baseline and this process was repeated.

Chronic Galantamine Administration: At 32-33 weeks of age, animals were randomly divided into four groups: control/vehicle, control/galantamine, SLE/vehicle, and SLE/galantamine. Animals were injected intraperitoneally with galantamine (Sigma, St. Louis, MO; 4mg/kg/day) (Pavlov et al., 2009) or the vehicle, normal saline, for 14 consecutive days.

Blood Pressure Measurements: At 34-35 weeks of age (following two weeks of galantamine injections), catheters were implanted into the left carotid artery as previously described (Mathis, 2015; Mathis, Broome, & Ryan, 2014a; Mathis et al., 2013, 2012, 2011; Mathis, Wallace, et al., 2014). Mean arterial pressure was then measured in conscious mice via pressure transducers for 1.5 hours for two consecutive days post-surgery. At the end of the study, anesthetized mice were

perfused with 2% heparinized saline and euthanized. Plasma and tissues (i.e., kidneys and spleen) were harvested and stored for biochemical analysis.

Autoantibody Measurements: Plasma double-stranded DNA (dsDNA) autoantibodies, an index of SLE disease severity, were quantified via commercial ELISA kits (Alpha Diagnostics), as previously described (Fairley & Mathis, 2017; Gilbert et al., 2014; Mathis et al., 2012; Mathis, Wallace, et al., 2014; Venegas-Pont et al., 2011).

Inflammatory Mediators: Upon euthanasia of anesthetized mice, the spleen was dissected from each mouse and was homogenized at 4°C in RIPA buffer. The renal cortex was dissected from the right kidney and was also homogenized in RIPA buffer. Aortas were stripped from the spine of each mice and then flash frozen in liquid nitrogen in order to quickly crush with a plastic plunger in order to homogenize with an appropriate amount of RIPA buffer (i.e., 8 times the weight of the aorta). Protein concentrations of the splenic, renal cortical and aortic supernatants were quantified via bicinchoninic acid assay (ThermoFisher).

Standard Western blotting techniques were performed as previously described using splenic, cortex-rich renal, and aortic homogenates (Fairley & Mathis, 2017) to measure TNF-α (catalog #sc-52746; Santa Cruz Biotechnology, Santa Cruz, CA); high mobility group box protein 1 (HMGB-1) (catalog #sc-26351; Santa Cruz Biotechnology, Santa Cruz, CA); and BAFF (catalog #MAB1357-100; R&D Systems, Minneapolis, MN), all well-accepted markers of pro-

inflammation. Proteins of interest were visualized using HRP-conjugated donkey anti-mouse, donkey anti-rabbit, or goat anti-mouse secondary antibodies, as appropriate. Western blots were imaged using the Chemi Doc MP Imaging System (Bio-Rad Laboratories, Inc; Hercules, CA) following chemiluminescent detection via Clarity Western ECL (Bio-Rad) and analyzed using ImageLab Software Version 5.1. Data are reported as volume intensity, which refers to the cytokine densitometry of the protein band normalized to total lane protein, which was determined by the stain-free total protein method as previously described (Gilda & Gomes, 2013). For TNF-α, both the 26 and 51 kDa bands were analyzed, the former comprising the transmembrane form and the latter constituting the active, secreted trimeric form (R. Smith & Baglioni, 1987). To emphasize the mechanistic role of renal inflammation upon blood pressure, only data from animals that also had blood pressure measurements were included in the analysis of renal pro-inflammatory cytokine measurements.

Indices of Renal Injury: Albumin excretion rate was determined following 24-hour urine collection via metabolic cages and quantification of urinary albumin by ELISA (Alpha Diagnostics) as previously described (Fairley & Mathis, 2017; Gilbert et al., 2014; Mathis, Broome, et al., 2014a; Mathis et al., 2013; Mathis, Wallace, et al., 2014; Venegas-Pont et al., 2011). Formalin-fixed sections of left kidney were embedded in paraffin, then sliced at 6 μm and stained with Periodic-Acid Schiff and Masson's Trichrome, and thirty glomeruli from each subject were blindly scored, as previously reported (Mathis, Wallace, et al., 2014).

Statistical Analysis: All data are calculated as mean ± standard error of the mean (SEM) and statistical analysis were performed using SigmaPlot 11.0 (Systat, Richmond, CA). Statistical differences (all $p < 0.05$) between multiple groups were determined by two-way ANOVA, with or without repeated measures, followed by the Holm-Sidak post-hoc test, as specified in the figures and accompanying figure legends.

RESULTS

Galantamine increases efferent vagus nerve activity: To confirm the ability of peripherally administered galantamine to potentiate efferent vagus nerve activity, anesthetized SLE and control mice were injected with galantamine (4 mg/kg ip), and the immediate response of the vagus nerve was recorded. Galantamine produced a robust increase in vagus nerve activity that was mirrored by a decrease in heart rate in control and SLE mice (n = 4/group; Fig. 1, B and D). In comparison, intraperitoneal injection of normal saline did not affect vagus nerve activity or heart rate in control or SLE mice (n = 3/group, Fig. 1, A and C).

Figure 1

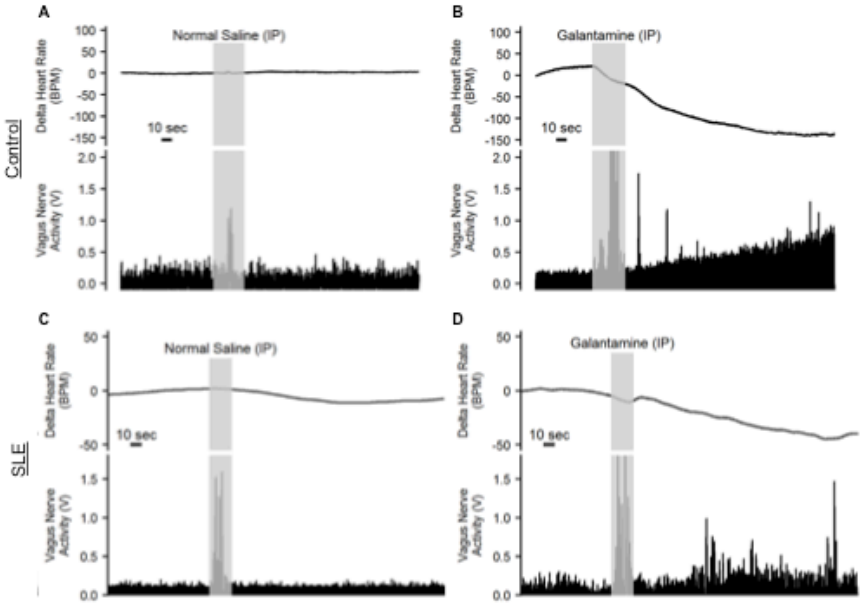


Fig. 1. Galantamine increases efferent vagus nerve activity. Representative vagus nerve activity (V, voltage) recorded from the cervical vagus, and corresponding heart rate (BPM, beats per minute) tracings at baseline and immediately after administration of normal saline or galantamine (4 mg/kg ip) in anesthetized control (A and B) and systemic lupus erythematosus (SLE, C and D) mice (n = 3–4/group).

Galantamine has no effect on body weight: SLE mice treated with vehicle and galantamine had higher body weight than control mice treated with vehicle and galantamine throughout the study ($P < 0.001$) (Fig. 2). In addition, body weight was significantly reduced throughout the study ($P < 0.001$). However, there was no significant interaction between treatment group and time ($P = 0.274$).

Figure 2.

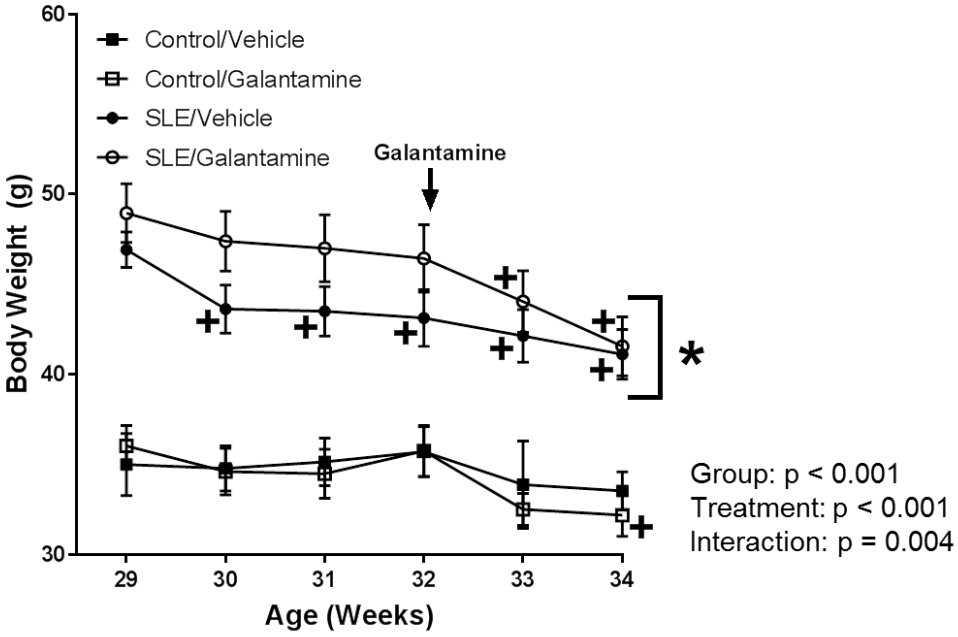


Fig. 2.Galantamine does not alter body weight. There was a natural significant reduction in body weight (g, grams) in all mice used in the study between weeks 32 and 34. Both vehicle- and galantamine-treated systemic lupus erythematosus (SLE) mice had greater body weight than control mice throughout the study. Body weight did not differ between vehicle-treated SLE mice and galantamine-treated SLE mice or between vehicle-treated control mice and galantamine-treated control mice. Values are presented as means ± SE. A two-way ANOVA with repeated measures was used to make statistical comparisons (n = 11–13/group).

Galantamine decreased plasma concentrations of dsDNA autoantibodies in female SLE mice: Plasma dsDNA autoantibodies are a commonly accepted diagnostic and prognostic indicator of the severity of SLE in both human patients and animal models. Female SLE mice

had elevated plasma dsDNA autoantibodies (3.0e5 ± 5.8e4 vs. 5.8e4 ± 1.8e4 activity units; P< 0.001) compared with controls (Fig. 3). Galantamine attenuated plasma concentrations of dsDNA autoantibodies in SLE mice (1.4e5 ± 3.0e4; P < 0.001) but had no significant effect in control mice (1.5e4 ± 4.1e3; P = 0.733).

Figure 3.

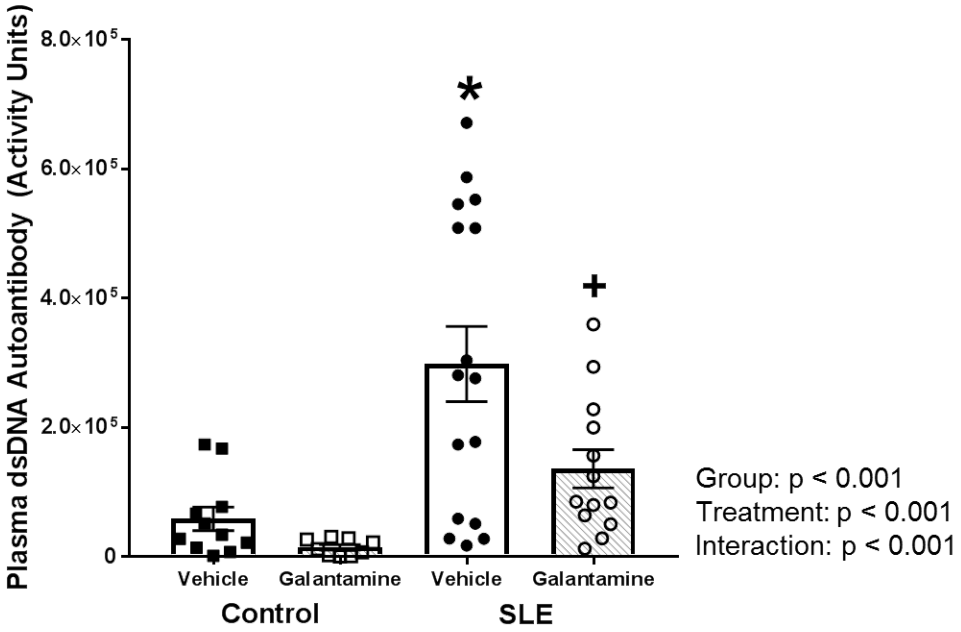


Fig. 3.Galantamine decreases SLE disease severity. Anti-double-stranded DNA (dsDNA) autoantibodies are specific to systemic lupus erythematosus (SLE) and used to diagnose the condition, as well as gauge the severity of disease. Female SLE mice have elevated anti-dsDNA autoantibodies in their plasma compared with control mice. Galantamine-treated SLE mice had attenuated anti-dsDNA autoantibody concentrations in their plasma compared with vehicle-treated SLE mice. Values are presented as means ± SE. A two-way ANOVA was conducted to

detect statistical differences. P values and accompanying symbols were determined using the results of Holm-Sidak post hoc analysis. (n = 11–13/group; *P vs. control/vehicle; +P vs. SLE/vehicle).

Galantamine reduces splenic cytokines in female SLE mice: As proposed, the cholinergic anti-inflammatory pathway results in a reduction in cytokine release from the spleen on stimulation of the vagus nerve. To confirm that galantamine alters splenic cytokines through modulation of the cholinergic anti-inflammatory pathway, we measured inflammatory mediators in the spleen following after galantamine therapy. Many of the splenic cytokines measured [e.g., both the 26-kDa (transmembrane) and 51-kDa (trimeric) forms of TNF-α and BAFF] appeared to be reduced after galantamine therapy in SLE mice; however, because of the variability of disease in the animals, significance was not reached following a two-way ANOVA (Fig. 4, A, B, and D). However, splenic HMGB-1 was elevated in SLE mice compared with controls (6.8e6 ± 1.3e6 vs. 1.0e6 ± 2.1e5 intensity units; $P < 0.001$) and decreased in galantamine-treated SLE mice compared with vehicle-treated SLE mice (2.0e6 ± 2.6e5 vs. 6.8e6 ± 1.3e6 intensity units; $P = 0.008$) (Fig. 4C). Galantamine had no effect on splenic HMGB-1 in controls (8.7e5 ± 1.7e5 intensity units; $P = 0.898$). Finally, splenic IL-1β was not different among control and SLE groups treated with galantamine or vehicle (Fig. 4E).

Figure 4A.

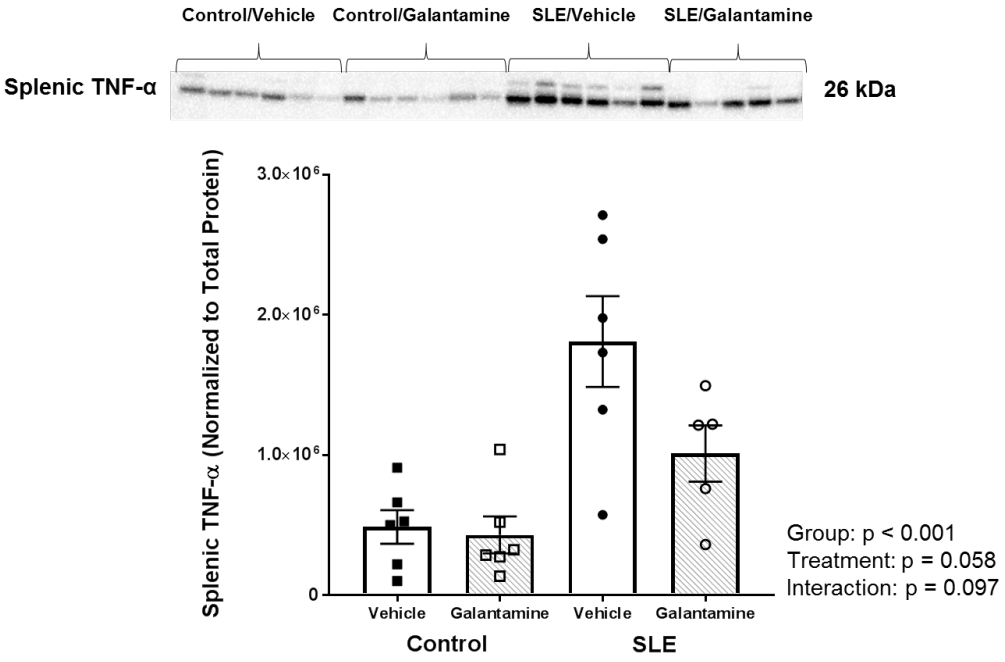


Figure 4B.

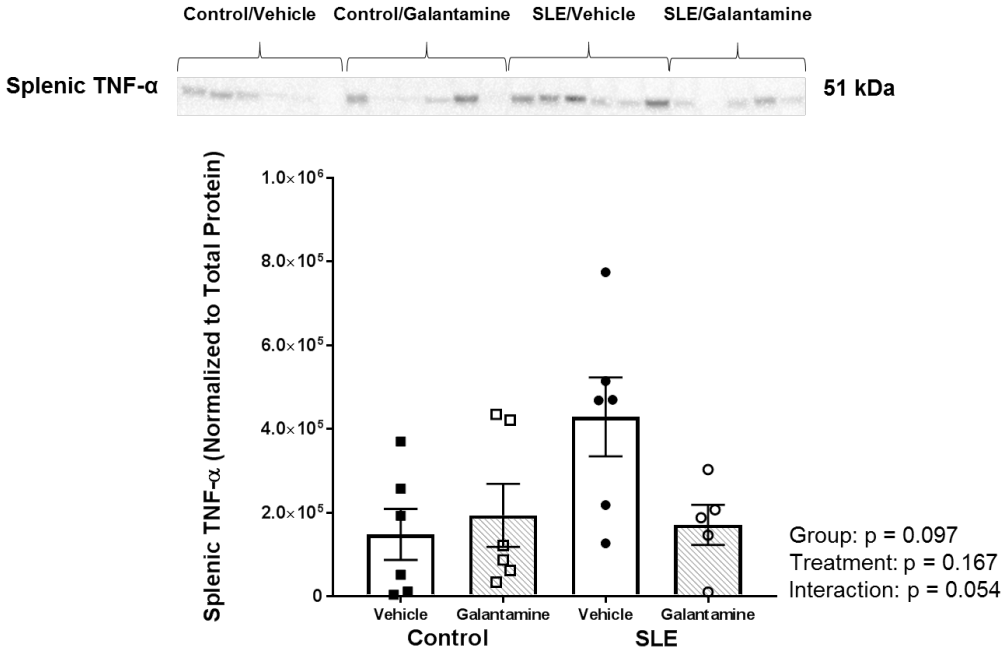


Figure 4C.

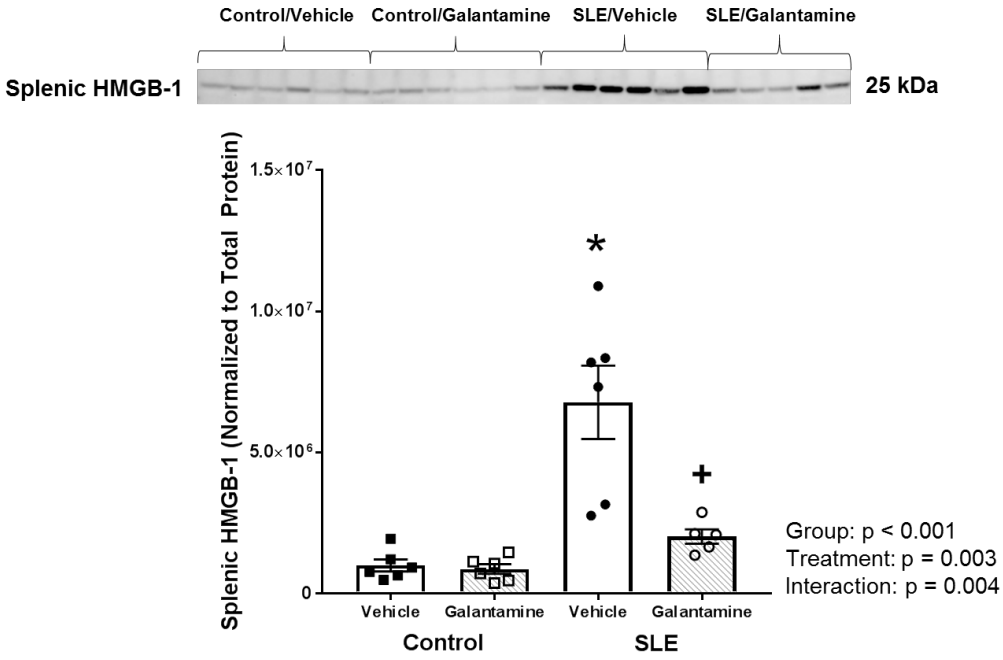


Figure 4D.

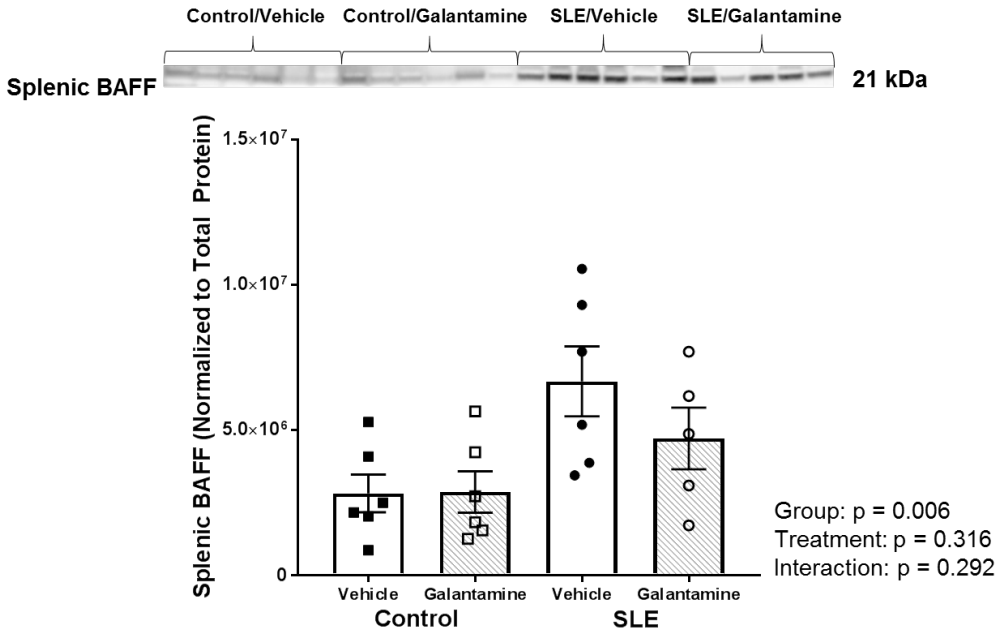


Figure 4E.

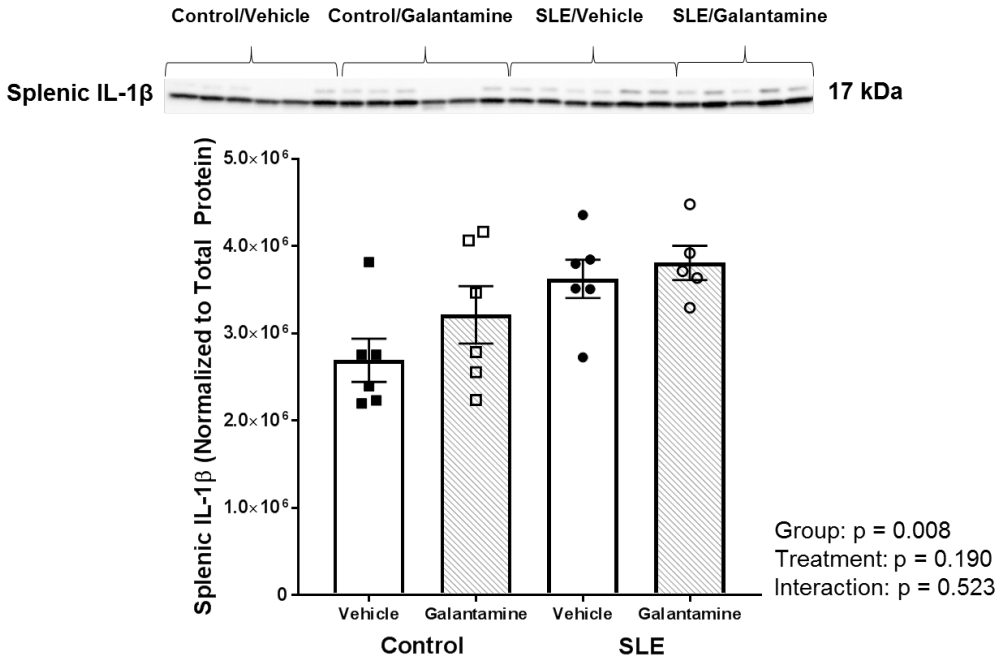


Fig. 4.Galantamine reduces splenic proinflammatory mediator in female SLE mice. A and B: Western blots of tumor necrosis factor (TNF)-α. C: high-mobility group box protein 1 (HMGB-1). D: B cell activating factor (BAFF). E: interleukin (IL)-1β in the spleen. Splenic TNF-α (at 26 kDa, the transmembrane form), TNF-α (at 51 kDa, the trimeric form), BAFF and IL-1β were not significantly altered. Splenic HMGB-1 was elevated in vehicle-treated systemic lupus erythematosus (SLE) mice compared with controls and galantamine markedly attenuated its expression. All values are presented as means ± SE. Statistical comparisons were determined using a two-way ANOVA. P values and accompanying symbols were determined using the results of Holm-Sidak post hoc analysis. (n = 5–6/group; *P vs. control/vehicle; +P vs. SLE/vehicle).

Galantamine prevents the rise in blood pressure and decreases renal injury typically observed in female SLE mice: To determine whether chronically enhancing the cholinergic anti-inflammatory pathway with galantamine effects SLE hypertension, we measured blood pressure following galantamine therapy. While control mice were normotensive, SLE mice were hypertensive at 34–35 wk of age (115 ± 2 vs. 141 ± 6 mmHg; P < 0.001) (Fig. 5A). Galantamine-treated SLE mice had ~12 mmHg lower mean arterial pressure than vehicle-treated SLE mice at 34–35 wk (128 ± 3 vs. 141 ± 6 mmHg; P = 0.024). Galantamine did not significantly affect blood pressure in control mice (124 ± 4 mmHg; P = 0.085).

Figure 5A.

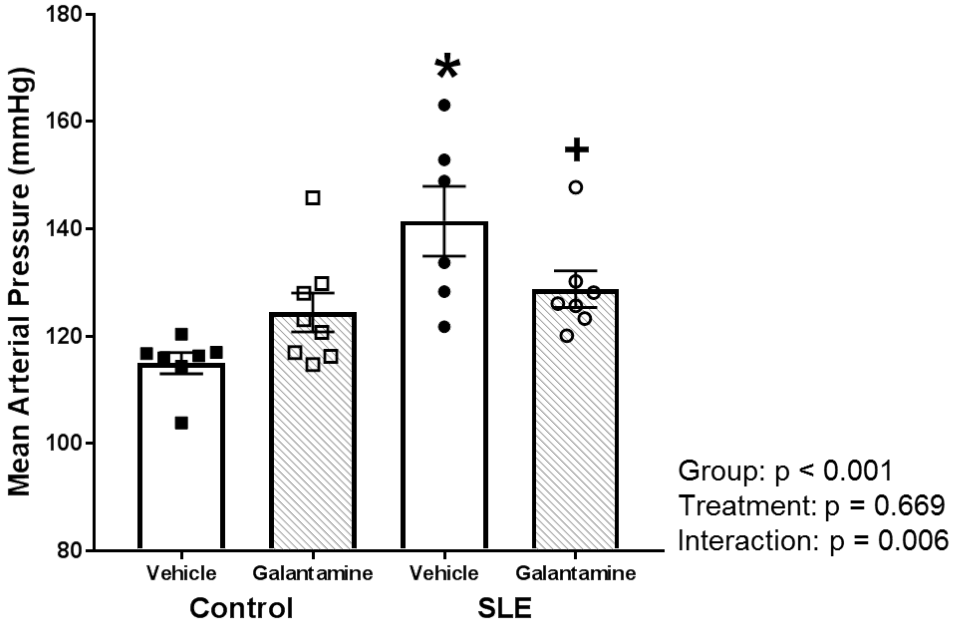


Figure 5B.

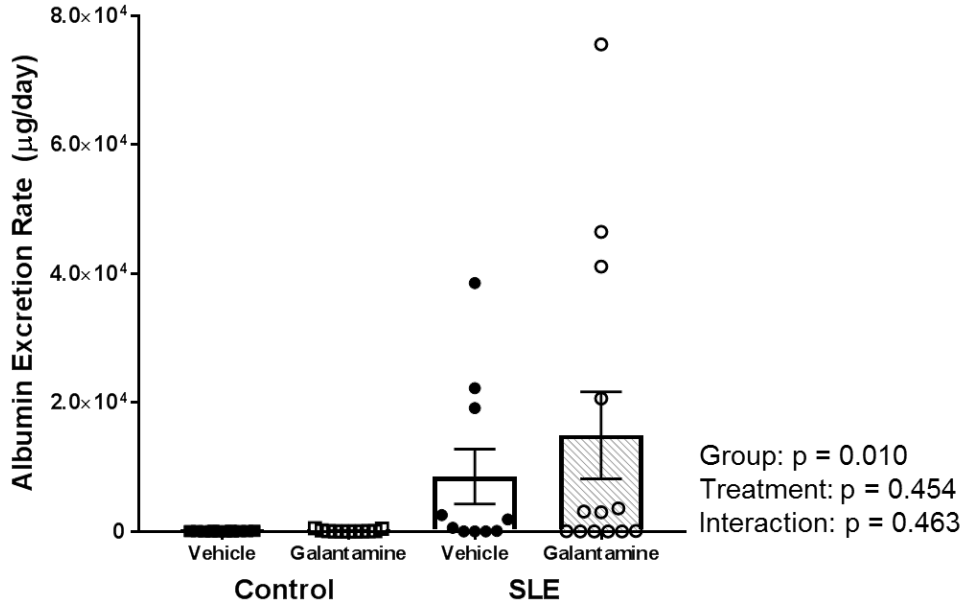


Figure 5C.

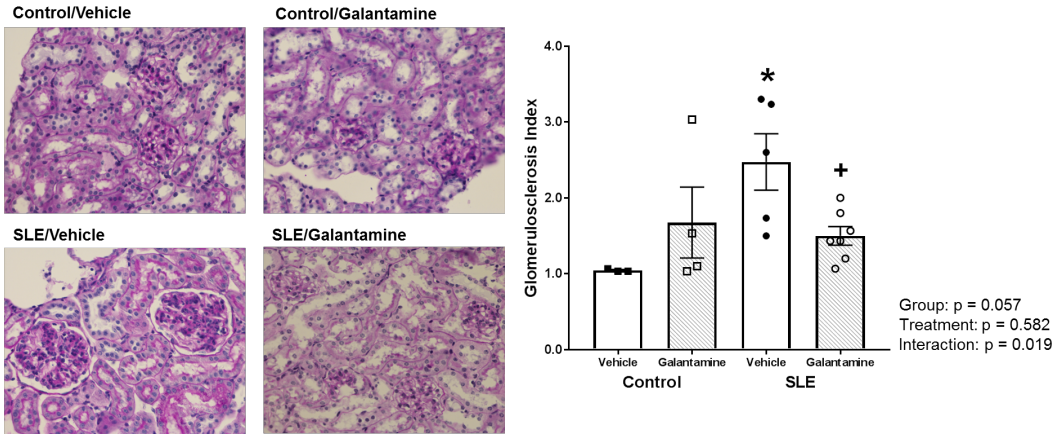


Fig. 5.Galantamine decreases blood pressure and dampens the severity of renal injury in female SLE mice. A: mean arterial pressure (mmHg) measured in conscious mice via indwelling carotid catheters. Systemic lupus erythematosus (SLE) mice had higher blood pressure compared with control mice. Galantamine-treated SLE mice were protected from the rise in blood pressure that

the vehicle-treated SLE mice experienced. Galantamine had no statistical effect on mean arterial pressure in controls. (n = 6–8/group) B: albumin excretion rate (AER) was based on results from albumin ELISA on urine and the amount of time spent in metabolic cages. Although there are obviously higher AERs in SLE mice, there was no significant difference among groups possibly due to variability of the disease. (n = 10–13/group) C: representative histological images and quantification of glomerulosclerosis in renal cortical glomeruli stained with Periodic Acid Schiff. Thirty glomeruli from each subject were blindly scored and assigned one of the following scores: 0: no glomerulosclerosis, hypercellularity, mesangial cell expansion, intact glomerular basement membrane; 1: 0 to 25% of the cross-sectional area is characterized by glomerulosclerosis, nodules, and hypercellularity; 2: 25 to 50% of the cross-sectional area is characterized by glomerulosclerosis, nodules, and hypercellularity; 3: 50–75% of the cross-sectional area is characterized by glomerulosclerosis, nodules, and hypercellularity; or 4: >75% of the cross-sectional area is characterized by glomerulosclerosis, nodules, and hypercellularity. SLE mice had increased glomerulosclerotic and hypercellular cross-sectional area compared with controls. Galantamine-treated SLE mice had decreased glomerulosclerosis compared with vehicle-treated SLE mice. (n = 3–7/group). All values are presented as means ± SE. Statistical comparisons were determined using a two-way ANOVA. P values and accompanying symbols were determined using the results of Holm-Sidak post hoc analysis. (*P vs. control/vehicle; +P vs. SLE/vehicle).

Although there were no significant differences in albumin excretion rate among treatment groups (Fig. 5B), SLE mice predictably had increased glomerulosclerotic injury compared with controls (P = 0.010). Galantamine-treated SLE mice had decreased renal glomerulosclerotic

injury compared with vehicle-treated SLE mice (P = 0.019) (Fig. 5C). Galantamine did not have a statistical effect on glomerulosclerosis in control mice (P = 0.212).

Galantamine decreases renal proinflammatory cytokine expression in female SLE mice: To determine whether a reduction in renal inflammation ultimately protected SLE animals from hypertension and renal injury, we measured inflammatory mediators in the renal cortex. When compared with control mice, SLE mice had higher renal cortical TNF-α, both in the 26-kDa (transmembrane) and 51-kDa (trimeric) forms (4.4e6 ± 7.5e5 vs. 6.7e5 ± 1.3e5 intensity units, P < 0.001 for 26 kDa; 4.4e6 ± 6.3e5 vs. 8.8e5 ± 2.4e5 intensity units for 51 kDa, P < 0.001), whereas galantamine-treated SLE mice had decreased expression of this cytokine compared with vehicle-treated SLE mice (2.4e6 ± 7.7e5 vs. 4.4e6 ± 7.5e5 intensity units, P = 0.028 for 26 kDa; 2.5e6 ± 6.5e5 vs. 4.4e6 ± 6.3e5 intensity units, P = 0.025 for 51 kDa) (Fig. 6, A and B). Galantamine had no effect on transmembrane or trimeric TNF-α in controls (1.2e6 ± 4.6e5, P = 0.476 for 26 kDa; 1.4e6 ± 5.8e5, P = 0.534 for 51 kDa).

Renal cortical HMGB-1 and IL-1β were elevated in SLE mice compared with controls (3.8e6 ± 1.1e6 vs. 9.6e5 ± 2.2e5 intensity units, P = 0.010 for HMGB-1; 1.7e7 ± 1.8e6 vs. 4.9e6 ± 5.7e5 intensity units, P < 0.001 for IL-1β). Galantamine did not have a significant effect on HMGB-1 or IL-1β in SLE (1.7e6 ± 4.6e5 intensity units, P = 0.068 for HMGB-1; 1.3e7 ± 2.8e6 intensity units, P = 0.083 for IL-1β) or control mice (2.6e6 ± 7.9e5 intensity units, P = 0.118 for HMGB-1; Fig. 6, C and E). Finally, renal cortical BAFF was not different (based on the results of the two-way ANOVA) among SLE and control groups treated with galantamine or vehicle (Fig. 6D).

Figure 6A.

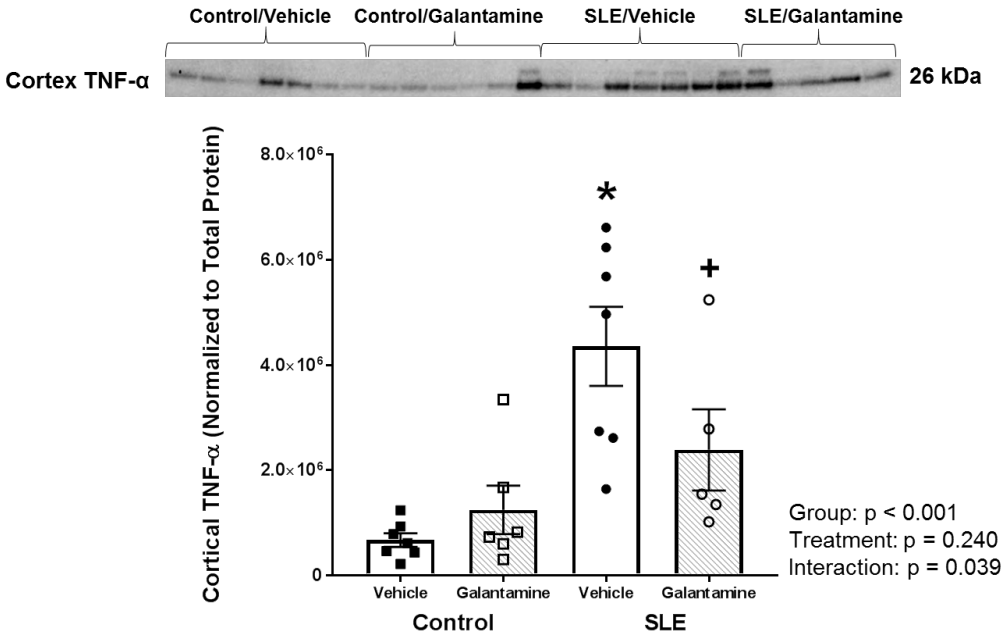


Figure 6B.

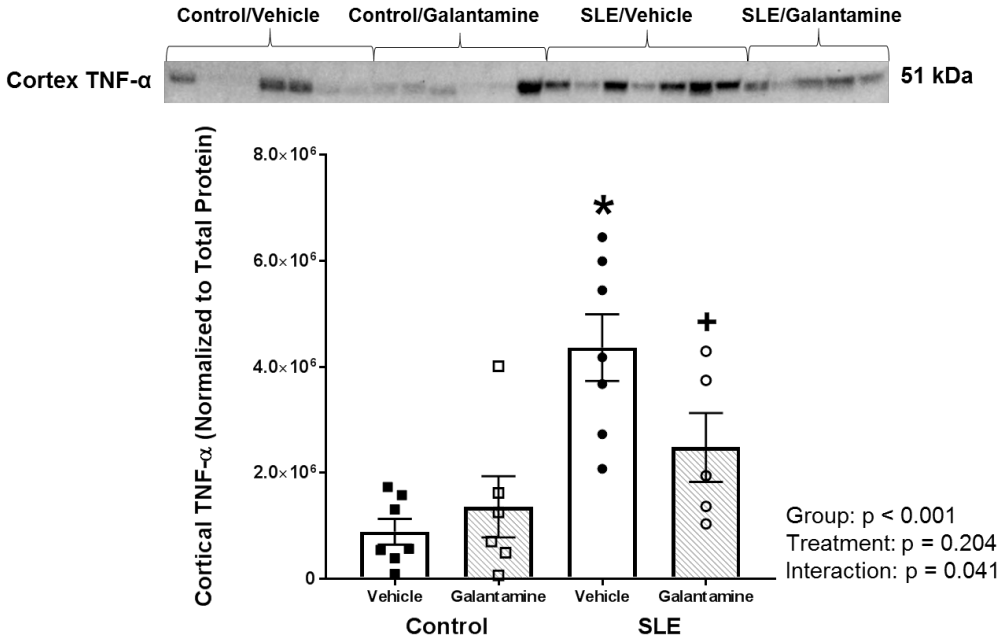


Figure 6C.

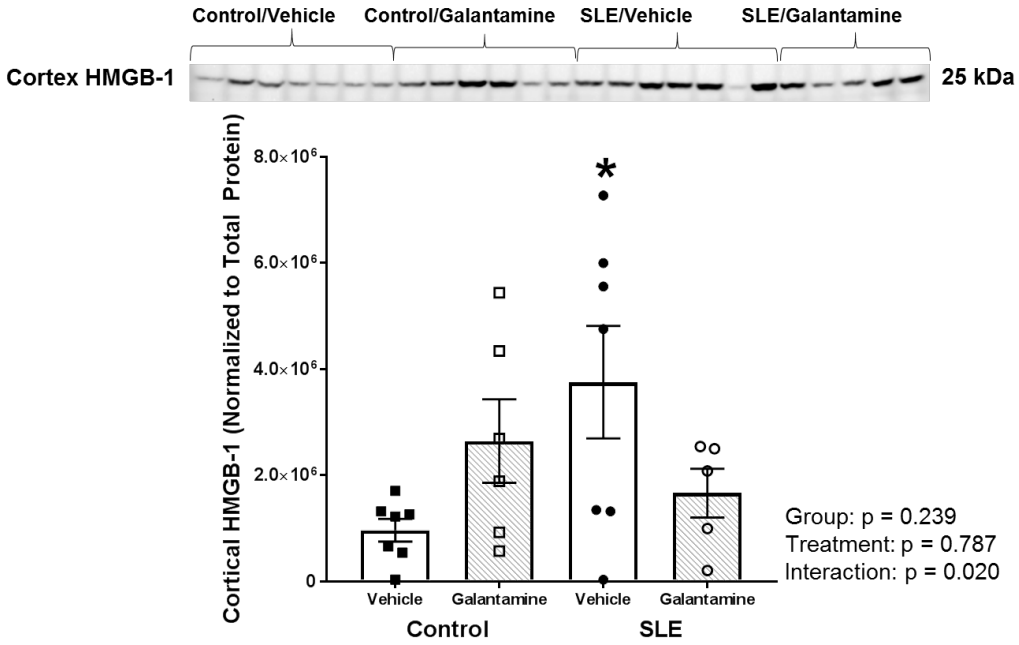


Figure 6D.

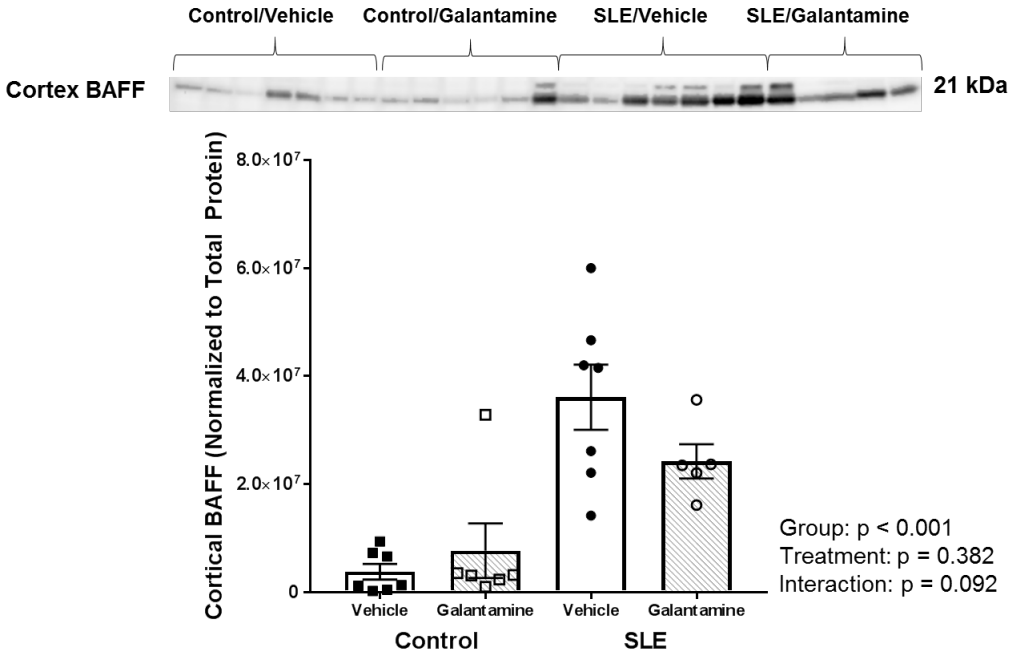


Figure 6E.

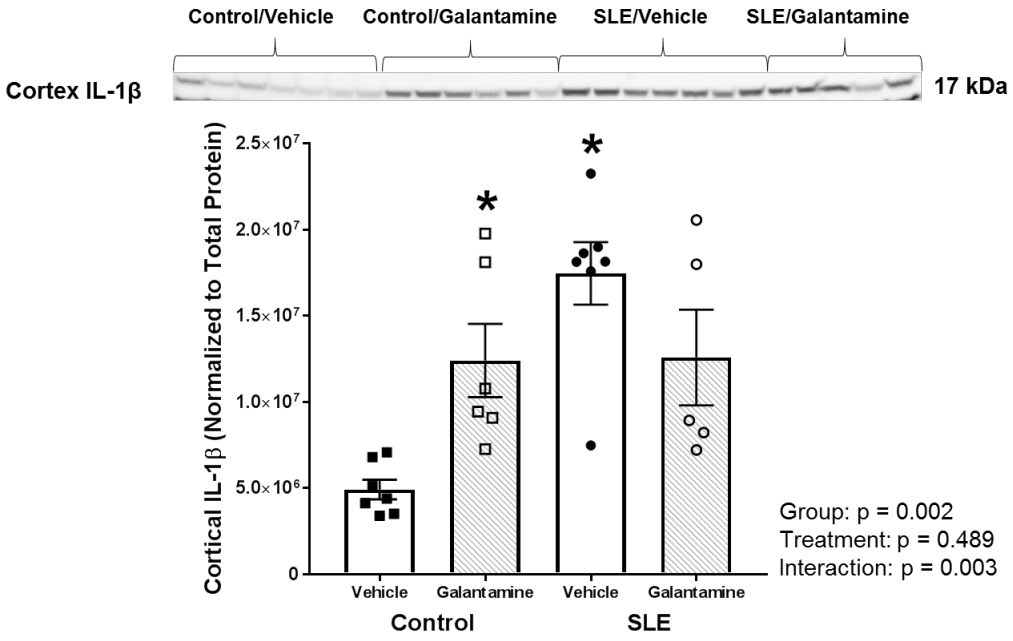


Fig. 6.Galantamine reduces renal cortical proinflammatory mediators in female SLE mice. A and B: Western blotting for tumor necrosis factor (TNF)-α. C: high-mobility group box protein 1 (HMGB-1). D: B cell activating factor (BAFF). E: IL-1β in the renal cortex. Renal cortical TNF-α (at 26 kDa, the transmembrane form) and TNF-α (at 51 kDa, the trimeric form) were elevated in vehicle-treated systemic lupus erythematosus (SLE) mice compared with controls, and galantamine markedly attenuated its expression. Renal cortical HMGB-1 and IL-1β were also increased in vehicle-treated SLE mice compared with controls; however, galantamine did not significantly alter expression. Statistical comparisons were determined using a two-way ANOVA. P values and accompanying symbols were determined using the results of Holm-Sidak post hoc analysis. (n = 5–7/group; *P vs. control/vehicle; +P vs. SLE/vehicle).

Galantamine does not affect aortic expression of TNF-α: Vascular inflammation is also implicated in the development of hypertension, so we investigated whether galantamine had any effect on vascular inflammation. Neither the transmembrane nor the trimeric forms of TNF-α were different between groups based on the results of a two-way ANOVA (Fig. 7, A and B).

Figure 7A.

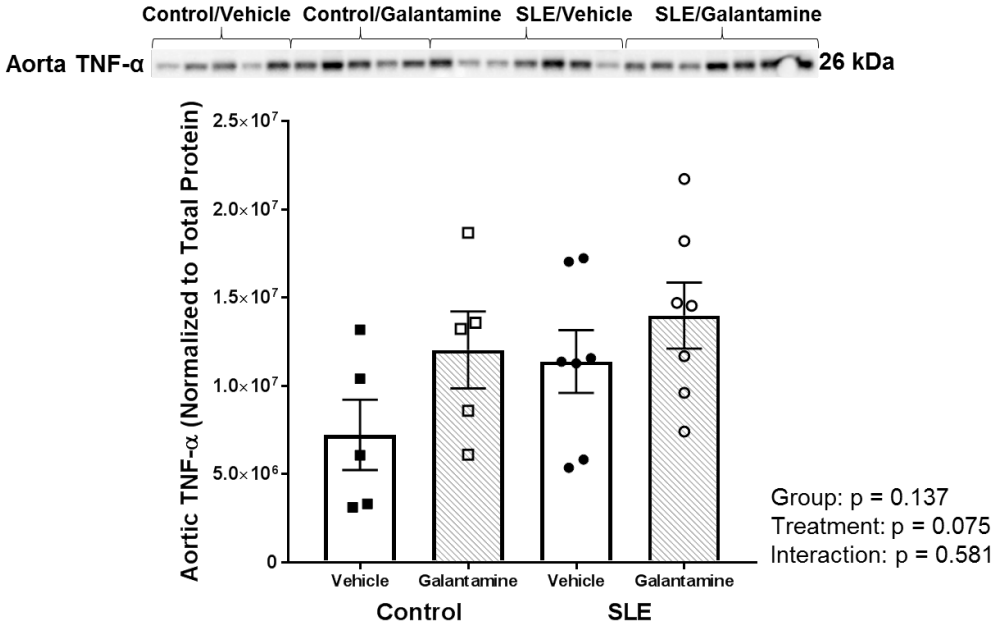


Figure 7B.

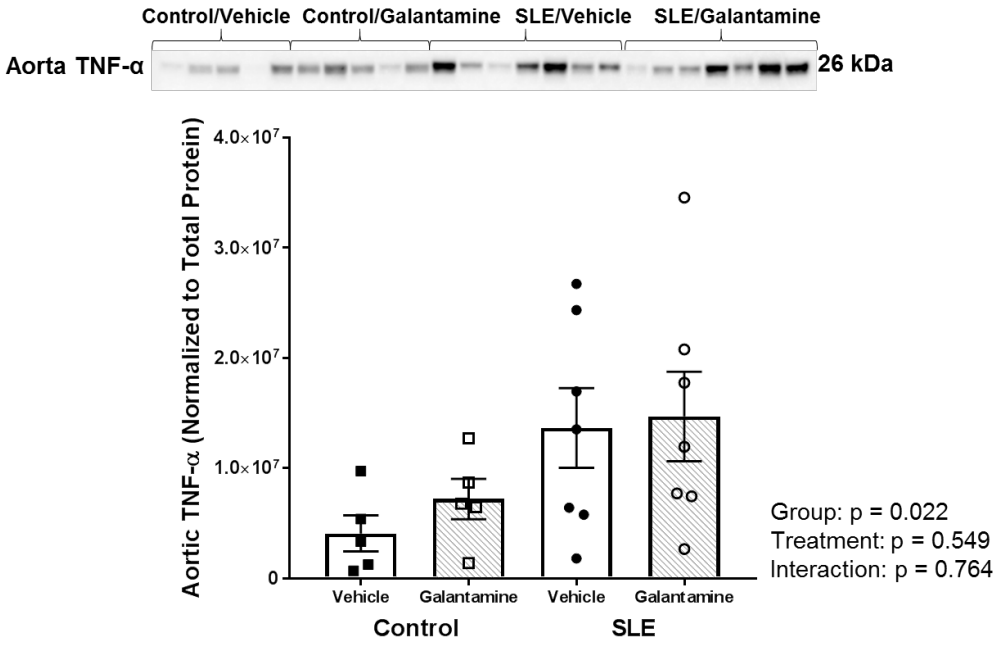


Fig. 7.Galantamine does not alter aortic proinflammatory mediators in female SLE mice. Western blot analysis of aortic TNF-α at 26 kDa (A) and 51 kDa (B) showed no differences between groups. SLE, systemic lupus erythematosus. Statistical comparisons were determined using a two-way ANOVA. P values and accompanying symbols were determined using the results of Holm-Sidak post hoc analysis (n = 5–7/group).

DISCUSSION

We demonstrated that 2 weeks of daily galantamine therapy 1) reduced SLE disease severity, 2) attenuated splenic inflammation and renal inflammation, and 3) reduced blood pressure and renal injury in SLE mice. Our findings suggest that the prevention of SLE hypertension may be the result of the effects of galantamine on systemic and renal inflammation, as well as SLE disease severity: 2 weeks of galantamine treatment decreased both splenic (Fig.

4) and renal cortical (Fig. 6) pro-inflammatory cytokine expression and significantly reduced dsDNA titer in SLE mice (Fig. 3). The results of this study indicate that potentiation of the efferent vagus nerve and enhancement of the cholinergic anti-inflammatory pathway may protect from renal inflammation, hypertension, and renal injury in SLE. Our findings increase curiosity of the protective effects of central cholinergic stimulation in diseases of chronic inflammation like hypertension and SLE.

Galantamine is a reversible acetylcholinesterase inhibitor that crosses the blood-brain barrier and prolongs acetylcholine expression to promote activity of cholinergic neurons (Geerts et al., 2005; Lilienfeld, 2006), especially clinically in Alzheimer's disease (Samochocki, 2003). Other clinical applications of galantamine include protection from glaucoma (Almasieh, Zhou, Kelly, Casanova, & Polo, 2010) and general neuroprotection (Barnes et al., 2000; Capsoni, Giannotta, & Cattaneo, 2002). Galantamine is known to potentiate efferent vagus nerve traffic because of its ability to prolong the life of acetylcholine at M1 muscarinic channels centrally (Samochocki, 2003). In rodents, galantamine was also demonstrated to act on central M2 muscarinic receptors to potentiate efferent vagus nerve activity (Ji et al., 2014). Others have suggested that galantamine may act peripherally by preventing cholinesterase activity in the vicinity of nicotinic receptors (Pohanka, 2014). We confirmed in this study that galantamine increases efferent vagus nerve activation (Fig. 1), and we propose that galantamine mediates its anti-inflammatory, therapeutic effects (also demonstrated in this study) by stimulating the cholinergic anti-inflammatory pathway and inhibiting downstream inflammation (Figs. 4 and 6). The cholinergic anti-inflammatory pathway is an intricate communicative mechanism that begins with efferent vagal transmission to the celiac ganglion, which prompts splenic nerve release of norepinephrine in the spleen, which activates specialized T cells to synthesize and release

acetylcholine. This T cell-generated acetylcholine then binds to α7-subunit of the nicotinic acetylcholine receptor (α7-nAChR) on splenic immune cells, ultimately inhibiting proinflammatory cytokine release (Andersson & Tracey, 2012; Bernik et al., 2002; Olofsson, Rosas-Ballina, Levine, & Tracey, 2012; Pavlov & Tracey, 2015; Kevin J. Tracey, 2002). The present study will help elucidate to the importance of this pathway in regulating inflammation in chronic inflammatory diseases.

The anti-inflammatory effects of galantamine being protective in murine SLE are not unusual, considering that immunomodulatory and immunosuppressive agents are the most common treatment for SLE patients as they delay the disease course and consequently lessen the severity of the many of the systemic complications of SLE (Ginzler et al., 2005; Navarra et al., 2011). The most common cause of death in SLE is cardiovascular disease (Giannelou & Mavragani, 2017), for which hypertension is a known risk factor. However, clinical trials for immunosuppressive drugs do not usually investigate the efficacy of these treatments against hypertension specifically, and other anti-inflammatory agents, especially exogenous glucocorticoids (Kasturi & Sammaritano, 2016), worsen hypertension (Lataro, Silva, Tefé-Silva, Prado, & Salgado, 2015). Donepezil, another acetylcholinesterase inhibitor that crosses the blood-brain barrier like galantamine, also improved the inflammatory profile in a cecal ligation and puncture mouse model of sepsis (Jeremias et al., 2016). Although the anti-inflammatory actions of acetylcholinesterase inhibitors are well documented (Gowayed, Refaat, Ahmed, & El-Abhar, 2015; Hanes & Olofsson, 2015; Ji et al., 2014), not much is known about the effects of chronic galantamine therapy on blood pressure regulation. An early study demonstrated that intravenous galantamine infusion results in an unusual acute hypertensive response in rats

(Chrusciel & Varagic, 1966); however, by contrast and as demonstrated in our present study, chronic galantamine administration has the opposite effect (Fig. 5A).

In our study, we discovered that galantamine therapy mediated an ~12 mmHg drop in blood pressure in the NZBWF1 disease model of SLE hypertension (Fig. 5A). Others have found that acetylcholinesterase inhibition prevented the development of hypertension and decreased plasma inflammatory markers in spontaneously hypertensive rats, although their treatments were prophylactically administered for a total duration of 16 wk (Lataro et al., 2015). In the present study, the implications of such a generous blood pressure difference after only 2 wk of treatment are exciting, since galantamine therapy began when inflammatory end-organ damage in the kidneys and blood vessels had already manifested in SLE mice. The reduction in renal injury that we discovered may help explain this drop in blood pressure, since renal inflammation promotes hypertension. In addition to decreasing both renal (and splenic) inflammation (Figs. 4 and 6), galantamine attenuated other autoimmune inflammatory processes in SLE mice (Fig. 3). Future studies may examine the effects of other clinically used acetylcholinesterase inhibitors in experimental hypertension to determine whether this therapeutic decrease is specific to galantamine or a property of other agents from its class.

In spontaneously hypertensive rats, donepezil decreased blood pressure and systemic inflammation while pyridostigmine (an acetylcholinesterase inhibitor that does not cross the blood-brain barrier) did not, indicating that central acetylcholinesterase inhibition specifically contributes to anti-inflammatory processes (Lataro et al., 2015). Since galantamine crosses the blood-brain barrier, we likewise found that central acetylcholinesterase inhibition mediates a powerful attenuation of autoimmune processes in SLE, as treatment with galantamine significantly reduced plasma dsDNA autoantibody concentration (Fig. 3), the most commonly

accepted diagnostic and prognostic indicator of the severity of human SLE (Förger, Matthias, Oppermann, Becker, & Helmke, 2004). The phenomenon that only two weeks of galantamine treatment significantly attenuated dsDNA autoantibody titer in SLE mice is exciting in terms of potential translational application to human SLE patients. Galantamine has additionally shown to be efficacious in nonobese diabetic (NOD) type 1 diabetic mice, another form of autoimmune disease resulting from antibodies produced against pancreatic islet cells (Hanes & Olofsson, 2015). After 2 weeks of daily injections, female NOD mice had a lower anti-insulin autoantibody titer, and both the incidence of hyperglycemia and diabetes was prevented in these mice (Hanes & Olofsson, 2015). Galantamine also decreased plasma, liver, and muscle inflammation through its central mechanism of action, as indicated by dose-dependent decreases in brain acetylcholinesterase activity, in the n5-streptozocin rat model of diabetes type II (Ali, El-Abhar, Kamel, & Attia, 2015).

Clinical studies have shown that SLE patients suffer from dysautonomia in the form of decreased efferent vagal tone, which manifests as decreased heart rate variability (Stein et al., 1995). Decreased heart rate variability is also noted in other chronic inflammatory states and may be indicative of a hypoactive cholinergic anti-inflammatory pathway (T. M. Cooper et al., 2015). For instance, in rheumatoid arthritis, patients with heightened heart rate variability indicating higher vagus nerve tone respond better to anti-inflammatory treatment (F. A. Koopman, van Maanen, Vervoordeldonk, & Tak, 2017). Our rationale for using galantamine in SLE is to correct a hypoactive or dysfunctional cholinergic anti-inflammatory pathway in the pathogenesis of lupus hypertension due to lessened vagus nerve firing, as galantamine potentiates vagus nerve firing centrally (Ji et al., 2014). The effects of galantamine on potentiating, and therefore functionally increasing efferent vagal outflow, call into mind

treatment modalities such as electrical vagus nerve stimulation, a novel therapeutic modality for conditions as varied as epilepsy, to rheumatoid arthritis (Zitnik, 2011), although pharmacological agents like galantamine and other acetylcholine-bolstering agents portend fewer cardiac side effects and are less invasive (Howes, 2014). Treatment could also be ceased more readily, if necessarily. However, reversible acetylcholinesterase inhibition is also known to cause systemic side effects, especially in the gastrointestinal system (Colovic, Krstic, Lazarevic-Pasti, Bondzic, & Vasic, 2013). No gastrointestinal side effects were noted in any of the animal groups treated with galantamine, and ultimately this manifested as no difference in body weight throughout the study (Fig. 2). In sum, our discovery that central vagus nerve potentiation using the acetylcholinesterase inhibitor galantamine protected from renal inflammation, hypertension, and renal injury in murine SLE shows that increasing cholinergic anti-inflammatory pathway activity may be protective in this autoimmune disease. This observation can also be taken to mean that the cholinergic anti-inflammatory pathway is hypoactive in SLE. Future studies will investigate dysfunction at other areas of the pathway in SLE, as well as possible deficiencies in other neuroimmune pathways mediated by the vagus nerve.

The results from this study are also promising in the context of therapeutics for essential hypertension. Chronic inflammation may be implicated in other etiologies in hypertension, as it is becoming increasingly known that systemic, low-grade inflammation is correlated with high blood pressure (Virdis, Dell'Agnello, & Taddei, 2014). Additionally, the loss of renal function, stemming from etiologies as diverse as SLE to diabetes, also accompanies and exacerbates longstanding hypertension (Stehouwer et al., 2002). Renal pro-inflammatory cytokine expression has been mechanistically linked to renal injury in the pathogenesis of hypertension. In fact, TNF-α in the kidney contributes to angiotensin-II-induced experimental hypertension (Zhang et al.,

2014) and inhibition of TNF-α with etanercept decreases renal inflammation and prevents hypertension in autoimmune- (Venegas-Pont et al., 2010) and angiotensin II-induced hypertension (Guzik et al., 2007), as well as in fructose- (Tran et al., 2009), high-salt- (Elmarakby et al., 2006), and deoxycorticosterone acetate-fed (Elmarakby et al., 2008) rats. Other anti-inflammatory agents yield therapeutic effects in hypertension, especially the immunosuppressive agent mycophenolate mofetil, an agent that inhibits T and B cell growth, which prevents salt-sensitive hypertension in angiotensin II-treated Sprague-Dawley rats while decreasing infiltrating renal immune cells (Rodríguez-Iturbe et al., 2001). A similar phenomenon was demonstrated in a mouse model of SLE, where mycophenolate mofetil reduces renal immune cells and renal injury while also preventing hypertension (Taylor & Ryan, 2017). Mycophenolate mofetil also has been shown to lower blood pressure in hypertensive rheumatoid and psoriatic arthritis patients after three months of treatment (Herrera et al., 2006). Clearly, anti-inflammatory agents mediate protective effects in both experimental animal models of hypertension and in hypertensive patients with autoimmune disease. Likewise, we discovered that galantamine treatment reduced glomerular injury in SLE mice (Fig. 5C), although it did not significantly impact urinary albumin excretion following drug administration (Fig. 5B). This finding may have been due to the time course of renal injury in SLE mice; perhaps the galantamine administration was able to decrease the severity of structural and glomerular injury in tandem with decreasing blood pressure, but glomerular basement injury continued to facilitate the loss of albumin in the urine. Additionally, the female NZBWF1 model of SLE presents variably in its phenotype, and it is not unusual for several mice from a cohort to undergo fulminant renal failure or other flare-ups of the disease, similarly to human lupus patients (Nakagawa et al., 2017); this phenomenon may help explain the lack of significance we obtained

in certain areas. Our study addresses both renal inflammation and injury in the pathogenesis and maintenance of hypertension, and supports the concept that targeting a higher, neural target that regulates inflammation (e.g., the vagus nerve) may be therapeutic, even in the context of established disease. Galantamine's central mechanism of action may help to elucidate novel targets in the treatment of both hypertension and SLE.

SUMMARY

The cholinergic anti-inflammatory pathway is one of several neuro-immune mechanisms that may be dysfunctional in the setting of lupus hypertension. If so, increasing activity of this endogenous mechanism may decrease end-organ inflammation as well as injury, and culiminate in a reduction in blood pressure. To this effect, galantamine was utilized for central cholinergic mechanism of action, which potentiates efferent vagus nerve transmission and would therefore chronically increase activity of the cholinergic anti-inflammatory pathway. The use of vagus nerve recordings in this study first establishes that galantamine increases efferent vagus nerve activity, while the chronic treatment protocol demonstrates therapeutic potential in the long-term control of blood pressure. The outcomes of this chapter, namely that chronic galantamine treatment does not adversely affect wild-type *NZW* mice, while ultimately benefitting the *NZBWF1* murine SLE strain, supports the overall hypothesis of this thesis that cholinergic anti-inflammatory pathway activity may be hypoactive or impaired in SLE, and correction of this deficiency, via pharmacological efferent vagus nerve potentiation, reduces blood pressure, renal inflammation, and renal injury in lupus hypertension.

CHAPTER III: UNILATERAL CERVICAL VAGOTOMY PREVENTS HYPERTENSION AND RENAL INJURY IN A MOUSE MODEL OF SLE

Introduction

Chronic inflammation, particularly renal inflammation, has been implicated in the development and maintenance of hypertension (Rodríguez-Iturbe, Franco, Tapia, Quiroz, & Johnson, 2012). Pro-inflammatory T cells are known mechanistic forces in the renal damage mediating hypertension and renal pro-inflammatory cytokines are also elevated in hypertensive patients (Virdis et al., 2014). This phenomenon yields comparison to the autoimmune disease systemic lupus erythematosus (SLE), where self-directed overactive T and B cells cause chronic inflammation. SLE patients demonstrate prominent renal inflammation or lupus nephritis and prevalent hypertension. Indeed, in certain cohorts up to 70% of young, female reproductive-age SLE patients are hypertensive when their age-matched healthy counterparts are protected from hypertension (J. M. Sabio et al., 2011). Furthermore, SLE patients commonly display dysautonomia in the form of dampened vagal tone (Aydemir et al., 2010; Maule et al., 1997), but it is unknown whether this contributes (directly or indirectly) to the renal inflammation and hypertension in SLE.

Efferent vagus nerve fibers participate in the reflex control of inflammation via immunoregulatory mechanisms like the cholinergic anti-inflammatory pathway (L. V. Borovikova, Ivanova, Zhang, et al., 2000b; Olofsson et al., 2012; Kevin J. Tracey, 2002). The outcome of this neuroimmune mechanism is increased acetylcholine synthesis and release by T cells in the spleen (M. Rosas-Ballina et al., 2011). This T cell-derived acetylcholine binds to its

α7 nicotinic acetylcholine receptors (α7nAChR) on other immune cells to reduce inflammation in the spleen, and therefore in peripheral organs across the organism (Bernik et al., 2002; Engel et al., 2015; K J Tracey, 2009).

Numerous acute studies have utilized vagotomy to demonstrate that the vagus nerve is instrumental to the anti-inflammatory actions of the cholinergic anti-inflammatory pathway. Following acute unilateral vagotomy, the anti-inflammatory response to a stimulus is typically abrogated, yielding heightened inflammation (L. V. Borovikova, Ivanova, Nardi, et al., 2000; L. V. Borovikova, Ivanova, Zhang, et al., 2000b; Ji et al., 2014; Mauricio Rosas-Ballina et al., 2016). There are currently a plethora of studies that investigate the role of the cholinergic anti-inflammatory pathway in models of chronic inflammatory diseases, from SLE to rheumatoid arthritis (Aydemir et al., 2010; Van Maanen et al., 2009); however, the long-term control of inflammation in a chronic vagotomized state is not well-known. Additionally, such studies have not yet been conducted in SLE to determine if interrupting vagal neurotransmission would impair immunoregulatory pathways. We hypothesized that chronic unilateral vagotomy would act similarly to acute unilateral vagotomy and dampen anti-inflammatory mechanisms, which would leave inflammation unabated and consequently worsen hypertension and renal injury in SLE mice.

Methods

Animals: Female NZBWF1 mice, a well-established murine model of SLE that is the first generation cross between the New Zealand Black and New Zealand White (NZW) strains, were used along with female NZW mice as controls as previously reported (Fairley & Mathis, 2017;

Mathis et al., 2013; Pham et al., 2018). All mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained on a 12-hour light/dark cycle in temperature-controlled rooms with access to food and water ad libitum. All animal studies were approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee (IACUC) and were in accordance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Unilateral Vagotomy Experiment: At 32 weeks of age, animals were separated into the following groups: Control/Sham, Control/Vagotomy, SLE/Sham, and SLE/Vagotomy. Mice in the vagotomy groups were anesthetized to make a 1 cm midline cervical incision, and then the right vagus nerve was isolated from the carotid artery and associated connective tissue bundle, ligated and transected. Mice in the sham procedure group were anesthetized and had the vagus nerve isolated, but not cut. At 35 weeks of age (3 weeks following unilateral cervical vagotomy), mice were implanted with carotid catheters as previously described to collect blood pressure data (Fairley & Mathis, 2017; Mathis et al., 2013; Pham et al., 2018). At the end of the study, mice were euthanized by cardiac perfusion with 2% heparinized saline and tissues were flash-frozen (plasma and kidney) or used fresh (spleen).

Index of disease severity: Anti-double stranded (dsDNA) autoantibodies, a hallmark of human SLE, was quantified in plasma samples obtained at 35 weeks of age via commercial ELISA kits (Alpha Diagnostic, San Antonio, TX) as previously reported (Pham et al., 2018).

Blood pressure measurements: In the two days following catheter surgery, mean arterial pressure (MAP) was measured for 1.5 hours per day in the morning when animals were least active using PowerLab (AD Instruments, Colorado Springs, CO) as previously reported (Fairley & Mathis, 2017; Mathis et al., 2013; Pham et al., 2018).

Urinary albumin determination: Urinary albumin was measured at 35 weeks of age via commercial ELISA kit (Alpha Diagnostic). Albumin concentrations were divided by the total urine volume rate to get albumin excretion rate (AER).

Preparation of splenocytes: In a subset of animals, freshly harvested spleen was weighed and single cell suspension of spleen was obtained in RPMI buffer by mashing the spleen through 100 μM cell strainer. After centrifugation the cells were suspended in ACK lysis buffer composed of 8.29 g NH4Cl, 1 g KHCO3, 37.2 mg Na2-EDTA (pH = 7.2-7.4) for 5 minutes to lyse the red blood cells followed by centrifugation and resuspension in FACS buffer (1%FBS, 2mM EDTA in PBS) at the concentration of about 106 cells/mL. The required amount of splenocytes were stained with Ghost Dye Red 710 (Tonbo Biosciences,San Diego, CA) for live gating during the flow cytometry analysis while remaining cells were used as negative control.

For flow cytometry analysis, splenocytes were incubated with Fc receptor blocking anti-CD16/32 antibody (BD Biosciences, San Diego, CA) for 15 min followed by staining with different cocktails of antibodies for 30 min at 40C to study immune populations like T cells, B cells, CD62+ cells, macrophages. The fluorochrome-conjugated antibodies used for surface staining were anti-CD3e FITC (clone 145-2C11) and CD8 PE (clone YTS169.4) (Invitrogen,

Carlsbad, CA) , CD4 PerCP (clone RM4-5), CD62 APC-Cy7(clone MEL-14), F4/80 APC (clone BM8) (eBioscience, San Diego, CA), CD19 PE-Cy7 (clone 1D3) (Tonbo). Following surface receptor labeling, cells were fixed with 2% paraformaldehyde and resuspended in FACS buffer for analysis.

Flow cytometry analysis: Data were acquired on a BD LSRII flow cytometer equipped with 488 and 633 nm excitation lasers in conjunction with FACS Diva acquisition software (BD Biosciences) and were analyzed by FlowJo version 10 software (Tree Star, Inc, Ashland, OR).

Measurement of Renal Inflammatory Mediators: Frozen right kidneys were thawed and renal cortices/medullas were isolated and homogenized with a RIPA buffer containing protease inhibitors. Primary antibodies for TNF-α, B cell-activating factor (BAFF), and high mobility group box (HMGB)-1 (Santa Cruz, Dallas, TX) were utilized as markers of inflammation as previously described. Western blots were imaged and subsequently analyzed with the ChemiDoc Imager and ImageLab software via total lane protein normalization (Bio-Rad, Hercules, CA) as previously reported (Fairley & Mathis, 2017; Pham et al., 2018).

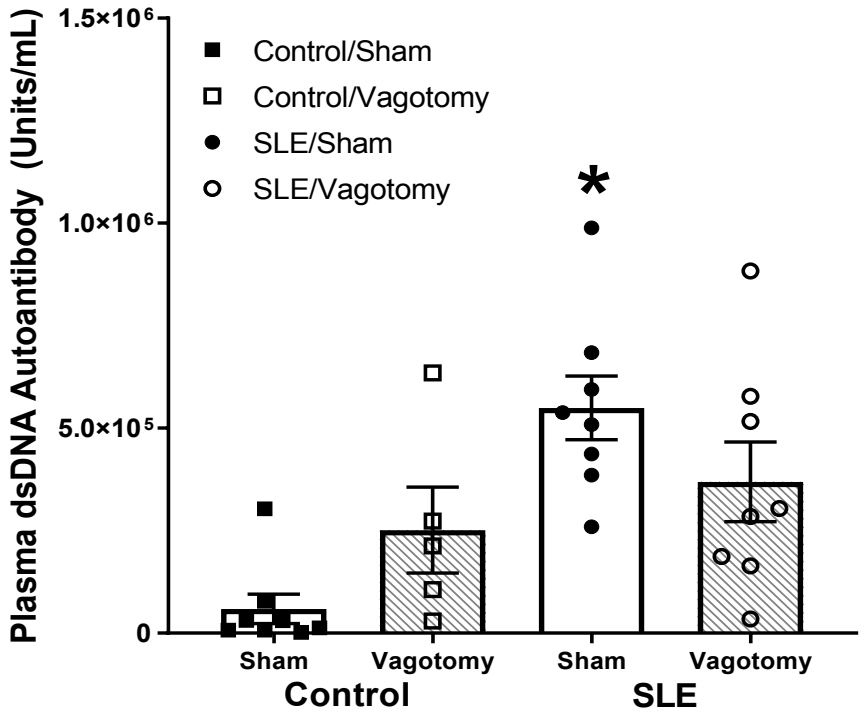
Nerve Activity Experiment: Vagus nerve recordings were performed on control and SLE mice as previously reported (Pham et al., 2018). A 10-minute baseline was recorded in Spike2 (Cambridge Electronic Design, Cambridge, UK) and then a single dose of galantamine (4 mg/kg dissolved in saline, IP; Sigma, St. Louis, MO) was injected. Post-injection vagus nerve activity was recorded for 1 hour.

Statistical Analysis: Two-way ANOVA with the Holm-Sidak post-hoc test was used to compare the four treatment groups. Unpaired t-test was used for comparison between two groups. All data were analyzed in SigmaPlot 11.2 (Systat, San Jose, CA) and were determined statistically different when $p<0.05$.

Results

Unilateral cervical vagotomy reduces blood pressure and renal injury in female SLE mice: Clinically and in the animal research setting, dsDNA autoantibodies are biomarkers for SLE. SLE mice had elevated dsDNA autoantibody titer (U/mL) compared to control mice (5.5e5 ± 7.8e4 vs. 5.9e4 ± 3.6e4; $p<0.001$; Figure 1A). Vagotomy did not significantly alter plasma anti-dsDNA autoantibodies in SLE mice (3.7e5 ± 9.7e4 vs. 5.5e5 ± 7.8e4; $p= 0.106$), nor in control mice (2.5e5 ± 1.0e5 vs. 5.9e4 ± 3.6e4; $p=0.130$).

Figure 1A.



In order to determine the effect of partial disruption of vagal neurotransmission on hypertension, we measured blood pressure in conscious mice 3 weeks post-unilateral vagotomy. SLE mice had higher MAP (mmHg) compared to control mice (158 ± 10 vs. 108 ± 4; $p<0.001$) (Figure 1B). Unilateral vagotomy prevented the rise in MAP in SLE mice (133 ± 4; $p=0.007$), while having no significant effect on MAP in control mice (121 ± 3; $p=0.105$). Body weight was not a confounding factor for the observed blood pressure differences. Overall SLE mice weighed more than controls, even at the conclusion of the study at 35 weeks ($p=0.006$; data not shown). Unilateral vagotomy had no effect on body weight in SLE ($p=0.316$) or control mice ($p=0.913$).

Sham-operated SLE mice had elevated AER (mg/day) compared to sham-operated controls (11113 ± 3233 vs. 106 ± 60; $p<0.001$; Figure 1C). Unilateral vagotomy blunted AER in SLE

mice compared to sham-operated SLE mice (3291 ± 1332 vs. 11113 ± 3233; $p<0.001$). There was not a significant difference in mean AER between sham-operated and unilateral vagotomized control mice (119 ± 65 vs. 106 ± 60; $p=0.996$).

Figure 1B.

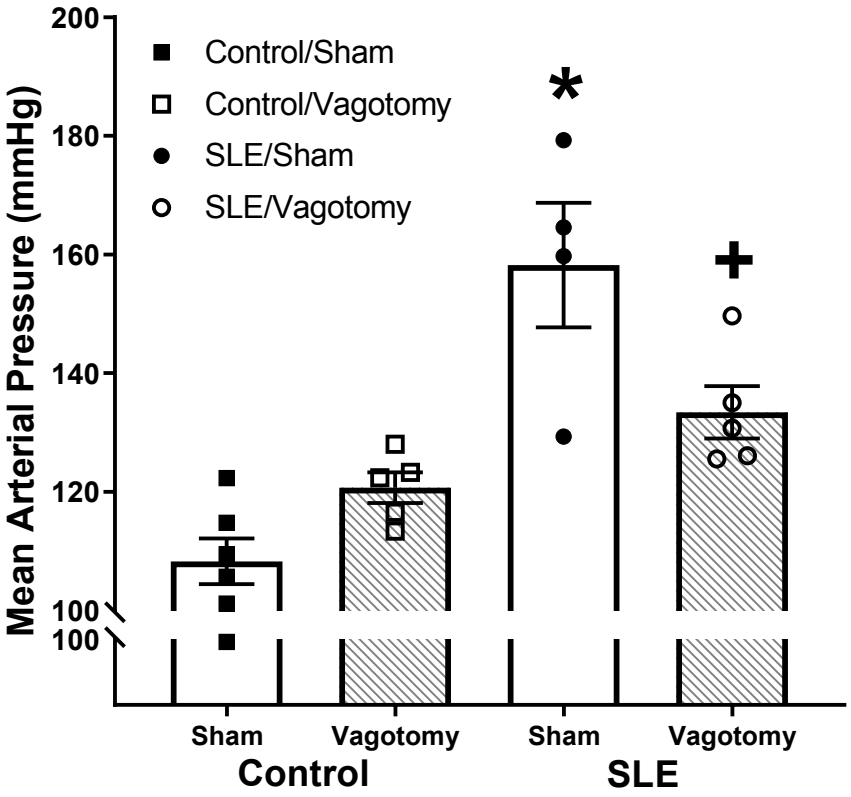
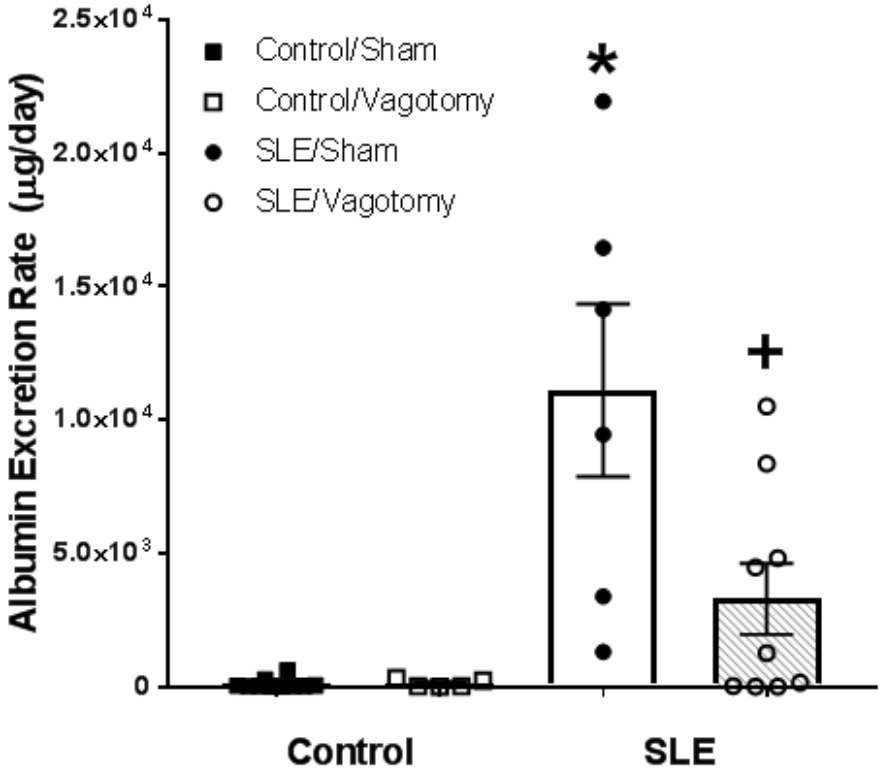


Figure 1C.



Glomerulosclerosis index was significantly higher in SLE mice compared to control mice (2.4 ± 0.4 vs. 1.1 ± 0.2; $p<0.001$; Figure 1D). Unilateral vagotomy blunted the glomerulosclerosis index in SLE mice (1.7 ± 0.3 vs. 2.4 ± 0.4; $p=0.004$), but had no effect on controls (1.1 ± 0.02 vs. 1.1 ± 0.2; $p=0.956$).

Figure 1D.

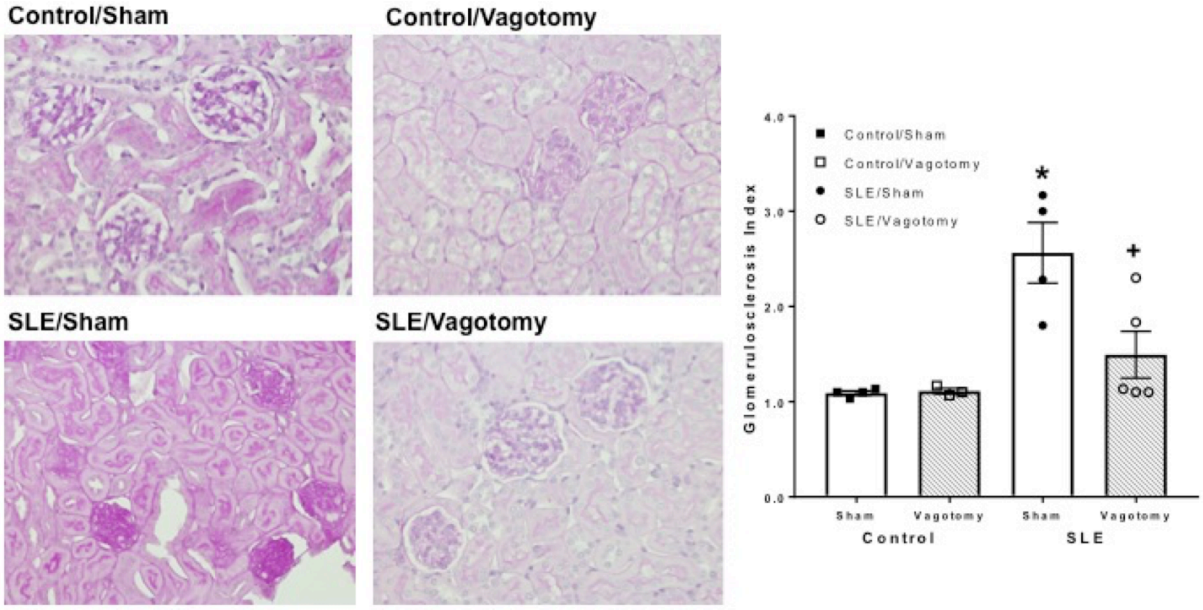


Figure 1: ***Unilateral vagotomy halts the rise in blood pressure and renal injury in SLE mice, without altering disease severity.*** A) SLE mice had elevated plasma anti-dsDNA autoantibodies compared to control mice ($p^* < 0.001$). Vagotomy did not significantly alter this parameter in SLE mice ($p=0.106$). Data for all mice ($n = 5$-8/group) were analyzed using a two-way ANOVA and symbols represent the results of post-hoc analysis. At 35 weeks of age, SLE mice had higher mean arterial pressure (MAP), albumin excretion rate (AER), and glomerulosclerosis index (GSI) compared to control mice ($p^* < 0.001$). B) Unilateral vagotomy protected from hypertension in SLE mice ($p+ = 0.007$) but had no effect on mean arterial pressure in control mice ($p=0.105$). Blood pressure data for all mice ($n = 4$-6/group) were analyzed using a two-way ANOVA and symbols represent the results of post-hoc analysis. C) Unilateral vagotomy reduced the SLE-induced increase in AER ($p+ < 0.001$), but had no effect in control mice unilateral vagotomized control mice ($p=0.996$). Albumin data for all mice ($n = 6$-10/group) were analyzed using a two-way ANOVA and symbols represent the results of post-hoc analysis. D) Unilateral vagotomy significantly decreased glomerular injury in SLE mice ($p+ = 0.004$), but not controls

(p=0.956). Glomerulosclerosis score was determined from slides stained with periodic acid Schiff (PAS) as previously reported and data for all mice (n = 3-5/group) were analyzed using a two-way ANOVA and symbols represent the results of post-hoc analysis.

Unilateral cervical vagotomy reduces splenic immune cell populations and renal inflammation in female SLE mice: After live gating, splenocytes were analyzed for different populations of immune cells such as CD4+ T cells (CD3+ CD4+), CD8+ T cells (CD3+ CD8+), B cells (CD19+), CD62L+ B cells, CD62L+ T cells, and macrophages (F4/80). Compared to the sham-operated SLE mice, unilateral vagotomized SLE mice demonstrated a significant reduction in splenic CD4+ T cells (24.53 ± 2.80 vs.15.58 ± 1.06; p=0.0138) (Figure 2).

Figure 2.

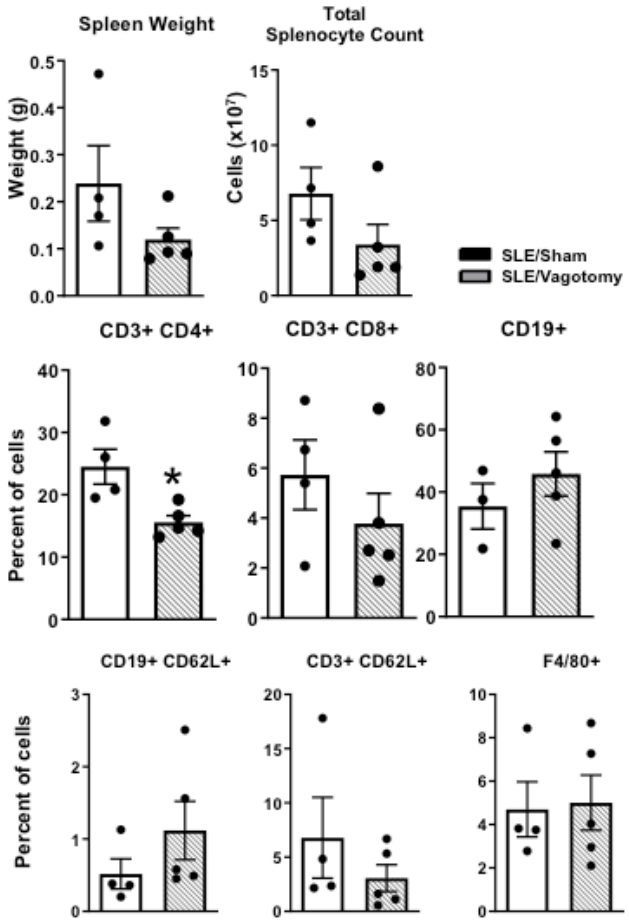


Figure 2: ***Unilateral vagotomy reduces inflammatory T cell populations in spleen from female SLE mice.*** Unilateral vagotomy decreased CD3+CD4+ T cells in the spleen of SLE mice. Data for all mice (n = 4-5/group) were analyzed using an unpaired t-test and p* < 0.001 vs. SLE/Sham group. Spleen weight and total splenocyte count are not significant (both p=0.159).

SLE mice had higher protein expression of TNF-α and BAFF in the renal cortex compared to controls (6.6e6 ± 2.5e6 vs. 2.4e6 ± 5.9e5, p=0.032; and 8.7e6 ± 2.7e6 vs. 2.4e6 ± 8.1e5, p=0.006; Figure 3A-3B). Unilateral vagotomy reduced renal cortical TNF-α and BAFF in SLE mice (2.1e6 ± 6.1e5, p=0.019 and 2.6e6 ± 4.9e5, p=0.006), but not controls (1.8e6 ± 6.3e5, p=0.779

and 2.0e6 ± 7.1e5, p=0.860). High mobility group box (HMGB)-1 was not significantly different amongst any of the groups (Figure 3C). The renal medulla yielded similar findings: SLE mice had higher protein expression of TNF-α and BAFF in the renal medulla compared to controls (2.5e6 ± 8.1e5 vs. 1.0e6 ± 3.4e5, p=0.026; and 7.6e6 ± 3.1e6 vs. 2.4e6 ± 7.2e5, p=0.026; Figure 3D-3E). Unilateral vagotomy reduced renal medullary TNF-α and BAFF in SLE mice (7.0e5 ± 1.6e5, p=0.009 and 2.5e6 ± 5.4e5, p=0.029), but not controls (6.3e5 ± 6.1e5, p=0.544 and 1.2e6 ± 4.7e5, p=0.559). Renal medullary HMGB-1 was increased in SLE mice compared to controls (2.9e6 ± 7.8e5 vs. 8.7e5 ± 3.0e5, p=0.009), but not significantly altered by unilateral vagotomy in SLE mice or controls (1.5e6 ± 4.7e5, p=0.061 or 4.2e5 ± 2.1e5, p=0.550; Figure 3F).

Figure 3A.

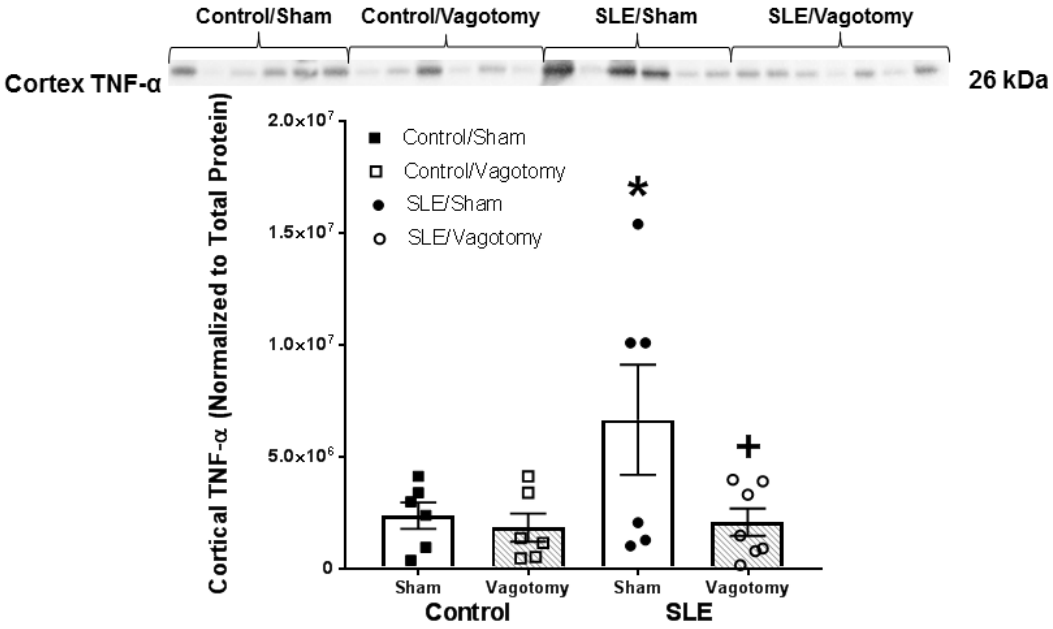


Figure 3B.

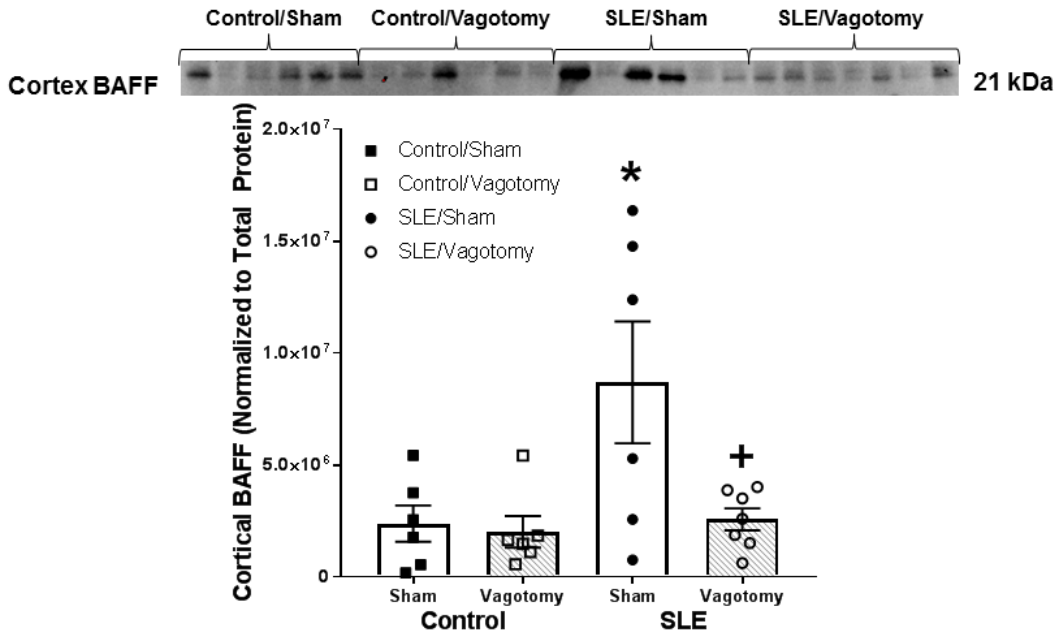


Figure 3C.

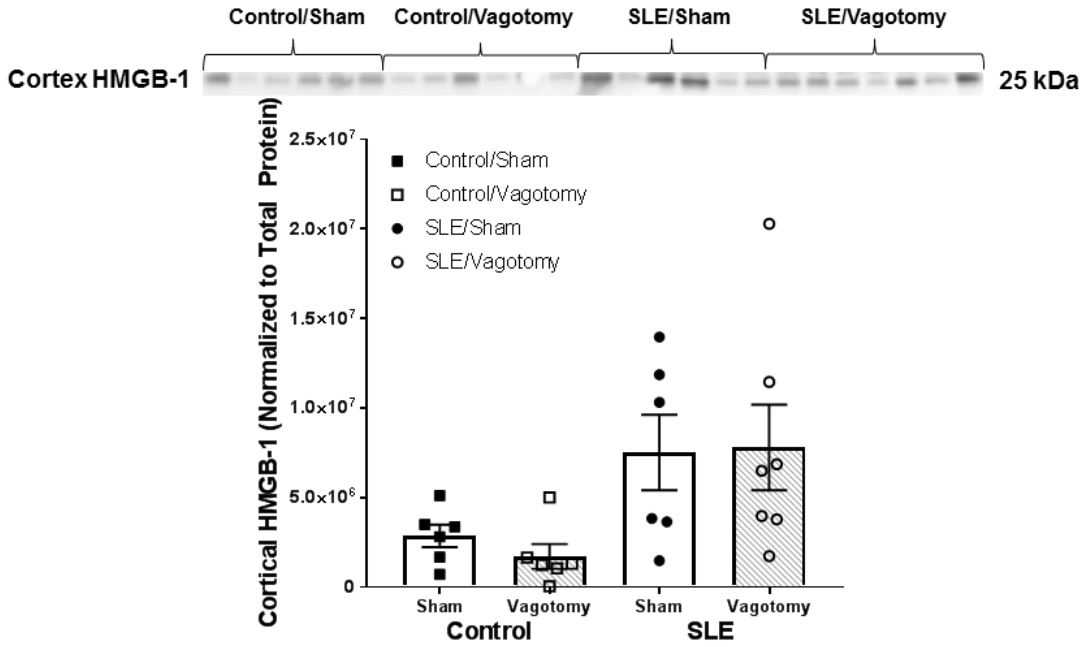


Figure 3D.

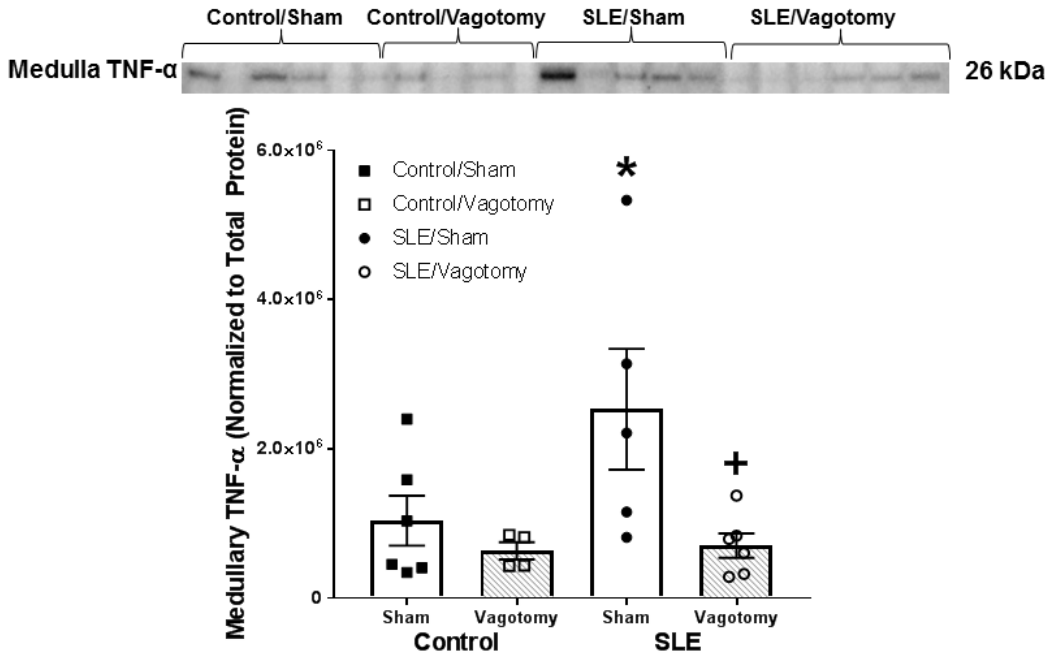


Figure 3E.

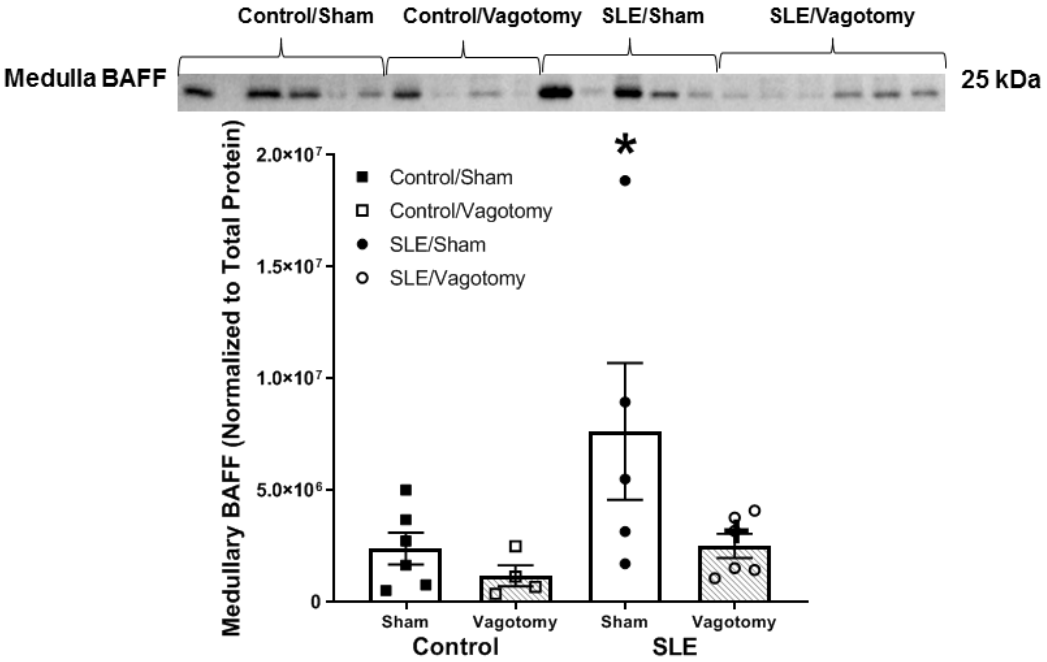


Figure 3F.

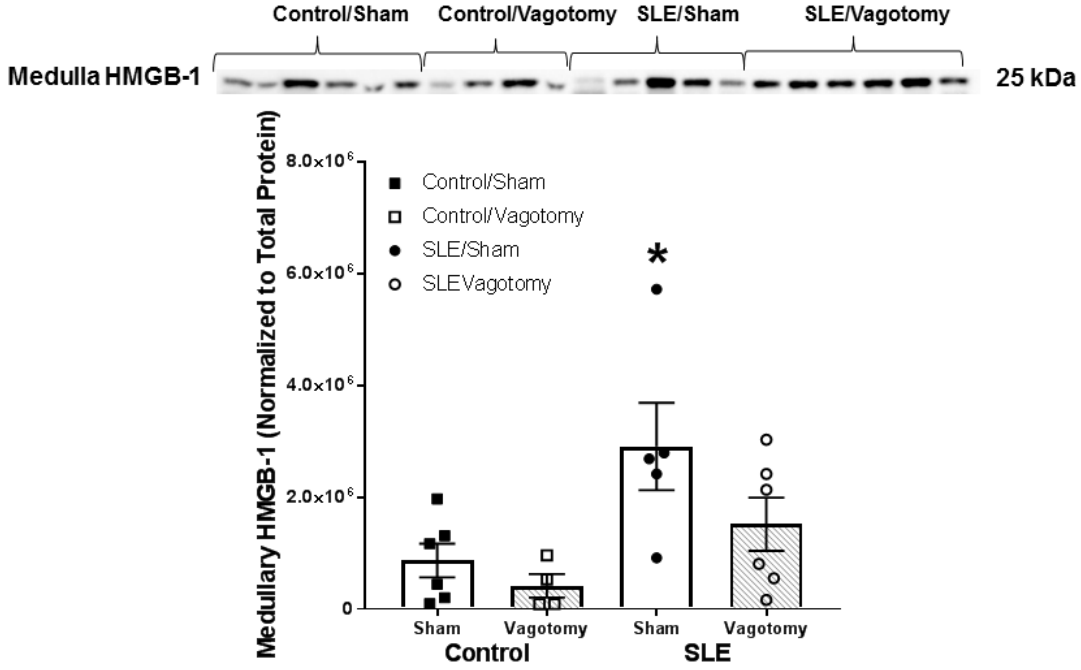


Figure 3: ***Unilateral vagotomy suppresses renal inflammation in female SLE mice.*** Protein expression of tumor necrosis factor (TNF)-α, B cell activating factor (BAFF), and high mobility group box (HMGB)-1 in the A-C) renal cortex and D-F) renal medulla of female SLE and control mice, as well as G) lung TNF-a. Unilateral vagotomy significantly blunted expression of renal cortical and medullary TNF-a and BAFF in SLE mice. Data for all mice (n = 6-7/group for cortex and n = 4-6/group for medulla) were analyzed using a two-way ANOVA and symbols represent the results of post-hoc analysis. p* vs. Control/Sham and p+ vs. SLE/Sham.

Contralateral vagus nerve compensates three weeks following vagotomy: Because the reduction in renal inflammation, blood pressure, and renal injury in SLE mice was not expected following unilateral vagotomy, we opted to study whether ligation of the right vagus nerve subsequently leads to compensatory increases in left vagus nerve activity. As shown previously,

intraperitoneal (IP) galantamine administration increased efferent vagus nerve activity and reduced heart rate in control mice (Figures 4A and 4C). After 3 weeks of unilateral vagotomy, there was a more pronounced decrease in heart rate and an enhanced increase in vagus nerve activity in the contralateral vagus nerve following IP galantamine in control mice (Figures 4B and 4D).

Figure 4A-D

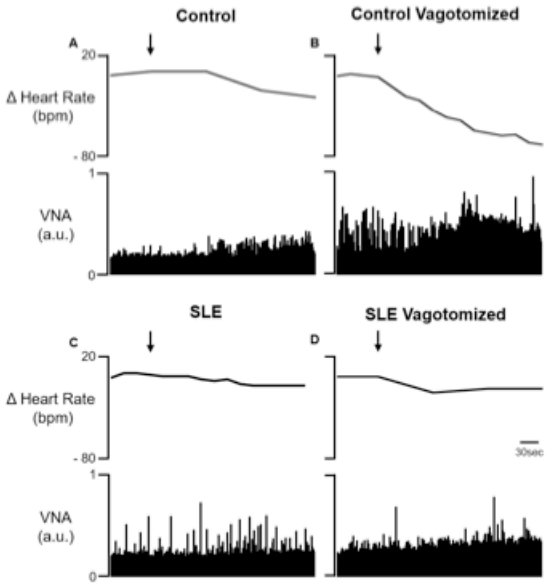


Figure 4: ***Contralateral vagus nerve compensates following three weeks of unilateral vagotomy*****.** Unilateral vagotomy increase galantamine responses of vagus nerve activity in control female animals. Representative examples of Vagus Nerve Activity (VNA) responses to IP injection of galantamine (4 mg/kg) in naïve female control mice and control mice that underwent 3 weeks of unilateral vagotomy. Arrows indicates time of galantamine IP injection (4 mg/kg dissolved in saline) (n = 3-4/group).

Discussion

In the current study, we tested the hypothesis that chronic right unilateral vagotomy would disrupt neurotransmission and worsen inflammation and outcome in SLE mice due to the inability of neuroimmune mechanisms like the cholinergic anti-inflammatory pathway to oppose this inflammation. Our main findings in this study contradicted our initial hypothesis and demonstrate that unilateral vagotomy is therapeutic in SLE mice. Chronic unilateral cervical vagotomy 1) blunted autoimmune-induced rises in blood pressure and renal injury in female SLE mice and 2) decreased indices of splenic and renal inflammation. A limitation of unilateral vagotomy is that the other vagus remains intact and could elicit a response. To determine if this was a factor in our study, we measured vagus nerve activity and observed that chronic unilateral vagotomy resulted in compensatory increases in the activity of the contralateral vagus nerve. This suggests that this enhanced efferent contralateral vagus activity following unilateral vagotomy bolsters immunoregulatory mechanisms like the cholinergic anti-inflammatory pathway and reduces renal inflammation and hypertension. Our support and rationale for this theory is the finding by Chen et al. that the contralateral intact vagus nerve compensates for hemodynamic and cardiovascular alterations following unilateral cervical vagotomy in rats (L. N. Chen et al., 2008). In that study, blood pressure significantly decreased following one week (denoted as "subacute") after unilateral cervical vagotomy (L. N. Chen et al., 2008). A similar phenomenon occurs with heart rate in unilateral vagotomized rabbits (Lund, Davey, Subieta, & Pardini, 1992). In our study we confirmed that the previously demonstrated increases in left vagus nerve activity following IP galantamine administration (Pham et al., 2018) are heightened three weeks following right vagotomy. This could explain why unilateral vagotomy is protective

in SLE mice; the cholinergic anti-inflammatory pathway remains activated by the enhanced neurotransmission through the contralateral vagus and effectively reduces renal inflammation.

Others have investigated the effects of vagus nerve stimulation (VNS) upon renal inflammation, most commonly in ischemia reperfusion injury models (Inoue et al., 2016b). The anti-inflammatory, protective effects of VNS also bolstered our rationale as to why unilateral cervical vagotomy would aggravate renal inflammation, even in a chronic setting such as SLE. It is well known that the vagus nerve innervates lymphoid organs like the spleen and bone marrow (Jung, Levesque, & Ruitenberg, 2017), but to our knowledge this is the first study to investigate the effects of chronic unilateral cervical vagotomy and link them to renal inflammation. Our flow cytometry data demonstrate a decrease CD4+ T cells in spleen after unilateral vagotomy, which supports a diminished inflammatory environment. Interestingly, there were no statistical differences between splenic macrophages suggesting that these cells do not play a role late in the disease in our model or in the absence of acute crisis. As for renal inflammation, the decreases in renal TNF-α, a cytokine implicated in the development and maintenance of hypertension, and BAFF, a cytokine belonging to the TNF ligand family, suggest that unilateral vagotomy may dampen systemic inflammation, albeit in a manner that does not lessen the severity of SLE, seeing as the plasma concentration of anti-dsDNA autoantibodies, a diagnostic and prognostic indicator of the severity of SLE, was unchanged by this intervention. Other investigators have elucidated the consequences of unilateral vagotomy in other organ systems, with varying outcomes. We had not anticipated that the transection of one vagus nerve would decrease renal inflammation, although there are studies in the literature that indicate that the vagus nerve may differentially affect inflammation in other organs like the lungs. For example, in one study

chronic unilateral cervical vagotomy (four weeks) decreased collagen deposition and alveolar injury in a mouse model of bleomycin-induced pulmonary fibrosis (Song et al., 2015). In another study, unilateral cervical vagotomy performed 2-4 weeks prior to capsaicin administration, reduced neurogenic inflammation in the ipsilateral bronchial tree in rats (Huang, 1993). It is possible that the time course may have resulted in systemic compensation for the transected nerve, as we have demonstrated. Another study indicates that unilateral cervical vagotomy protects from T cell egression and the development of angiotensin II-hypertension in male mice (Carnevale et al., 2016). These data directly reflect our findings in hypertensive SLE mice. On the other hand, there are studies that demonstrate that a chronic vagotomized state leads to increased inflammation and exacerbation of disease processes in the cardiovascular and gastrointestinal systems (Di Giovangiulio & Bosmans, 2016; Li-Sha et al., 2017; van Westerloo et al., 2005). The time course of vagotomy in relation to an inflammatory stimulus or disease induction appears to be critical to the outcome. Further studies are warranted to solidify the role of vagal efferents in regulating inflammation in health and chronic inflammatory diseases.

The cholinergic anti-inflammatory pathway is a novel neuroimmune mechanism that has been reviewed extensively and serves as an important regulator of inflammation (Mathis, 2015; Olofsson et al., 2012). In the context of chronic inflammatory diseases, it is unclear as to whether this pathway is functioning properly or impaired. In detail, the cholinergic anti-inflammatory pathway may be stimulated at the level of the efferent vagus nerve and efferent vagal transmission to the celiac ganglion causes activation of the splenic nerve, which induces the release of norepinephrine within the spleen. Splenic nerve-derived norepinephrine binds β2-adrenergic T cells in the spleen, which prompts a subset of CD62L positive T cells to synthesize

and release acetylcholine. This T cell-derived acetylcholine then binds to α7nAChR on splenic immune cells to prevent the release of pro-inflammatory cytokines. Many studies involving the cholinergic anti-inflammatory pathway have utilized unilateral or bilateral cervical vagotomy as a means of determining whether the vagus nerve was required for anti-inflammatory responses to an acute stressor, typically lipopolysaccharide or cecal ligation and puncture (L. V. Borovikova, Ivanova, Nardi, et al., 2000; L. V. Borovikova, Ivanova, Zhang, et al., 2000b). The current study is the first to use unilateral cervical vagotomy to test the importance of the vagus nerve and cholinergic anti-inflammatory in controlling inflammation in SLE. Our intriguing data from this study and previous studies indicate blunted vagus nerve activity in female SLE mice (Pham et al., 2018), which suggests that the vagus nerve should be a focus for novel therapeutic targets for SLE in the future.

Ultimately, the results of the current study may lead towards additional discoveries in the pathogenesis of SLE, especially insofar as neuroimmune interactions are considered. There are notable limitations, such as the use of a global intervention (i.e., transecting both afferent and efferent vagal nerve fibers), but the strength of the functional data we obtained make it clear that there are complex physiological mechanisms that arise and impact inflammation following unilateral cervical vagotomy. Targeted future studies could utilize agents such as capsaicin to selectively denervate unmyelinated afferent vagal fibers, although myelinated afferent vagal nerve fibers would remain intact (Bernal-Mizrachi et al., 2007). Agents such as cinnamaldehyde activate the afferent vagus through transient receptor potentiating activating (TRPA) 1 channels and may be useful for dissecting functional outcomes of increasing afferent vagal activity (Nassenstein et al., 2008). We have previously conducted studies in our lab that use

pharmacological agents to increase efferent vagus nerve activity (Pham et al., 2018). More studies are needed to fill the knowledge gap concerning the link between the vagus nerve, the cholinergic anti-inflammatory pathway, and immune system dysfunction in chronic inflammatory diseases such as hypertension and SLE.

SUMMARY

The vagus nerve is an integral component of the cholinergic anti-inflammatory pathway, as well as the HPA axis; the efferent vagal fibers mediate the actions of the former mechanism, while the afferent fibers help transmit sensory information to the latter. This chapter employed unilateral cervical vagotomy as a chronic intervention, which was anticipated to decrease activity of both the cholinergic anti-inflammatory pathway and the HPA axis, and therefore increase renal inflammation, renal injury, and blood pressure. Ultimately, the outcomes of this study were unanticipated, as unilateral cervical vagotomy resulted in decreased blood pressure, renal inflammation, and renal injury in SLE mice. Future studies may utilize localized vagotomy surgeries targeting vagus nerve branches that are more specific to either the cholinergic anti-inflammatory pathway or the HPA axis, as the cervical vagotomy likely culminated in widespread, systemic consequences. Nevertheless, the results from this experiment indicate that there may be complex compensatory responses to neuro-immune pathway disruption, and that such responses may be worth further investigating in the interest of elucidating novel mechanisms that contribute toward hypertension.

CHAPTER IV: CHRONIC INTRAPERITONEAL CURCUMIN AGGRAVATES INFLAMMATION AND RENAL INJURY IN MURINE LUPUS

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by inflammatory renal injury and prevalent hypertension, and disproportionately affects minority females of reproductive age (Benedek, 2019; Pons-Estel et al., 2017; Rahman et al., 2000; J. M. Sabio et al., 2011). SLE is typically managed with synthetic corticosteroids, such as prednisone and prednisolone, which are accompanied by a plethora of unwanted side effects, including weight gain and psychiatric manifestations, and may promote elevated blood pressure due to its mineralocorticoid effects (M Petri et al., 2014). As the main cause of mortality in SLE is cardiovascular disease, it is imperative to prevent hypertension, an independent risk factor for adverse cardiovascular events, in this autoimmune disease process.

In response to treatment-resistant hypertension and antihypertensive agents that possess unwanted side effects, natural products and "nutraceuticals" have emerged as adjunct treatments (Houston, 2013). Curcumin, the active product of the rhizome and cooking spice turmeric, has been extensively studied for its anti-inflammatory (Chainani-Wu, 2003), antioxidant (Motterlini, Foresti, Bassi, & Green, 2000), and anti-cancer properties (Tomeh, Hadianamrei, & Zhao, 2019). By attenuating both inflammation and oxidative stress, curcumin may be therapeutic in hypertension due to overwhelming evidence of inflammatory contributions to hypertension. Systemic (and especially renal) inflammation has been implicated in the organ damage that leads to heightened blood pressure. Pro-inflammatory plasma cytokines have been correlated with high

blood pressure, especially in older patients (Bautista et al., 2005). Renal pro-inflammatory cytokines have especially been correlated to renal injury and hypertension in rodent models (Rodríguez-Iturbe et al., 2012; Zhang et al., 2014). Oxidative stress is another factor that has been implicated in the development and maintenance of hypertension (Touyz, 2004).

In addition to its antioxidant and anti-inflammatory properties, curcumin has been demonstrated to exhibit corrective influences on the hypothalamic-pituitary-adrenal (HPA) axis. Curcumin normalized HPA axis activity in a mouse model of depression induced by chronic stress, even when compared to the antidepressant imipramine (Xu et al., 2006). Furthermore, curcumin may directly activate the "gut-brain" anti-inflammatory axis by activating vagal afferents (Dou et al., 2018), leading to central downregulation of acetylcholinesterase and enhancement of choline acetyltransferase in the brain (Ishrat et al., 2009; Yadav et al., 2011), which would culminate in peripheral anti-inflammatory effects (Bonaz & Pellissier, 2016). *In vitro* curcumin application increased TRPA1 channel activation on mouse sensory vagal neurons (Leamy, Shukla, McAlexander, Carr, & Ghatta, 2011), which are known to facilitate HPA axis activity. Another study demonstrated that curcumin activates vagal afferents through interactions with ion channels (Qiao, Dou, Hu, & Dai, 2019). Due to curcumin's activating effects on vagal afferents, along with its anti-inflammatory and antioxidant properties, we hypothesized that a chronic curcumin administration of 4 weeks duration would reduce renal inflammation, renal injury, and blood pressure in the *NZBWF1* murine SLE model.

METHODS

Animal Model: Female SLE (*NZBWF1)* and control (*NZW/LacJ*) mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 5-6 weeks of age. All mice were maintained on a 12-hour light/dark cycle in temperature-controlled rooms with access to food and water *ad libitum.* Mice were monitored starting at 29 weeks of age, an age at which SLE mice have already developed autoantibodies and renal disease. All animal studies were approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee (IACUC) and were in accordance with National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*.

Curcumin Administration: Powdered curcumin with a purity of >94% (Sigma) was suspended in sesame oil (Sigma) at a dose of 50 mg/kg animal body weight (Avasarala et al., 2013). Animals were injected intraperitoneally for 28 consecutive days with the curcumin suspension or sesame oil as vehicle in the following experimental groups (n=4-5/group): Control/Vehicle, Control/Curcumin, SLE/Vehicle, SLE/Curcumin. For a separate group of animals, curcumin or sesame oil as vehicle were intraperitoneally injected three times weekly, for 14 weeks, starting at 20 weeks of age (n = 2-3/group): Control/Vehicle, Control/Curcumin, SLE/Vehicle, SLE/Curcumin.

Mean Arterial Pressure (MAP): At 34 weeks of age, we surgically implanted catheters into the left carotid artery as previously described (Fairley & Mathis, 2017; Gilbert et al., 2014; Mathis, Broome, & Ryan, 2014b; Mathis et al., 2013; Pham et al., 2018; Venegas-Pont et al., 2010).

MAP was measured for 1.5 hours in the following 2 days and reported as an average of the last hour of blood pressure monitoring between the two days (PowerLab).

Renal Injury: Albustix (Siemens) were used weekly for an approximate determination of urinary albumin content, with a threshold of 300 ng/dl being our criterion for the development of albuminuria. A more quantitative measure of urinary albumin excretion was determined from commercial albumin ELISA kits (Alpha Diagnostic), using urine samples from 34 weeks of age.

Splenic, Renal, Lung, and Liver Markers of Inflammation: We separated the renal cortex from medulla as previously described (Fairley & Mathis, 2017). Splenic, renal, lung, and liver tissues were homogenized with RIPA buffer that was dissolved with a protease inhibitor tablet (Sigma). We used a BCA assay (company) to determine the protein concentration in the homogenates, then calculated volumes to use 100 ng protein per lane in each western blot. Primary antibodies for TNF-α, HMGB-1, IL-6, MCP-1, were utilized. All western blots were imaged and analyzed using the ChemiDoc Imager and ImageLab 5.1 software, respectively (Bio-Rad).

Statistical Analysis: All data are calculated as mean ± standard error of the mean (SEM) and statistical analysis were performed using SigmaPlot 11.0 (Systat, Richmond, CA). Statistical differences ($p < 0.05$) between multiple groups were determined by two-way ANOVA, with or without repeated measures, followed by the Holm-Sidak post-hoc test, as specified in the accompanying figure legend.

RESULTS

Curcumin does not affect body weight in SLE mice. (Figure 1) SLE mice weighed, on average, 11.5 g more than control mice ($p = 0.008$). Curcumin-treated SLE mice did not differ in weight from vehicle-treated ($p = 0.285$). No difference in body weight due to curcumin was observed in control mice ($p = 0.878$).

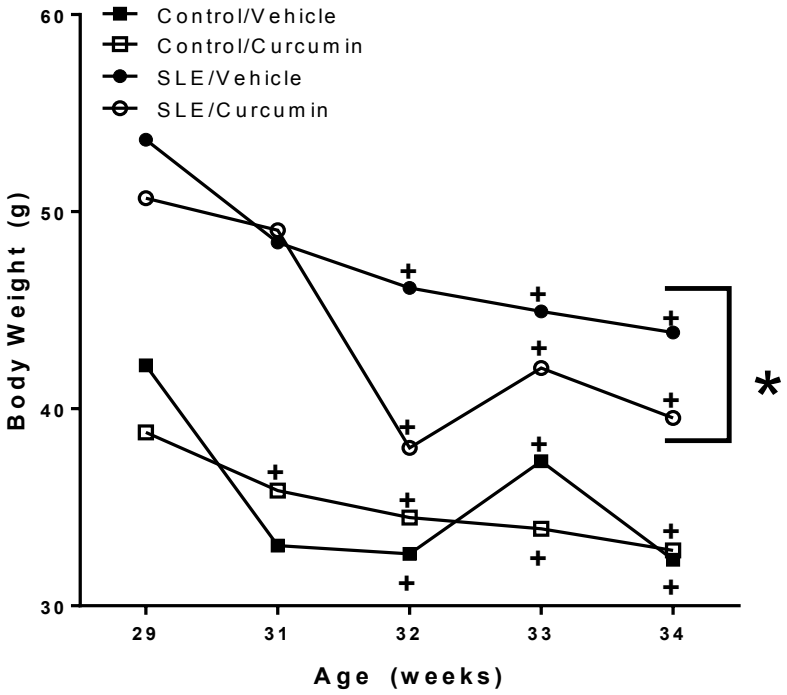


Figure 1. Curcumin did not affect body weight. SLE mice weighed more than controls ($p = 0.008$). Curcumin did not change body weight in SLE mice ($p = 0.285$). Values are presented as mean ± SEM. A two-way ANOVA with repeated measures was used to make statistical comparisons, and p values (all $p < 0.05$) and accompanying symbols were determined using the results of Holm-Sidak post-hoc analysis. n = 4-5/group. $p^* \leq 0.001$ SLE vs. Control. $p+ \leq 0.05$ Week 29 vs. +

Curcumin does not impact dsDNA titer in SLE mice. (Figure 2) Compared to control mice, SLE mice had elevated plasma anti-DNA autoantibodies (13670 ± 4681 vs. 5802 ± 2927 activity units, p = 0.001). Curcumin-treated SLE mice did not differ from vehicle-treated in plasma concentrations of this pathognomonic autoantibody (p = 0.899).

Figure 2

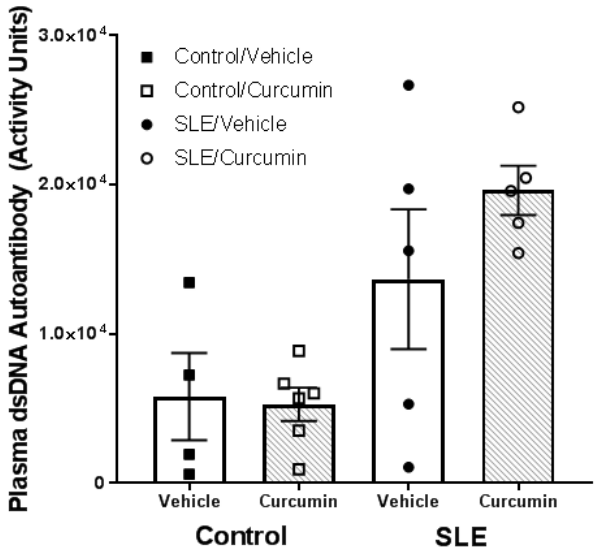


Figure 2. Curcumin did not alter anti-dsDNA titer. SLE mice had elevated plasma anti-dsDNA autoantibodies compared to controls (p = 0.001) and curcumin did not increase their titer in SLE mice (p = 0.353). Values are presented as mean ± SEM. A two-way ANOVA with repeated measures was used to make statistical comparisons, and p values (all $p < 0.05$) and accompanying symbols were determined using the results of Holm-Sidak post-hoc analysis. n = 4-5/group

Curcumin increases renal cortical and medullary pro-inflammatory cytokines. (Figure 3A) Splenic TNF-α was increased in SLE mice compared to controls (2.6e7 ± 4.4e6 vs. 1.6e7 ±

2.0e6; p = 0.006) and elevated in curcumin-treated SLE mice (4.9e7 ± 7.1e6 vs. 2.6e7 ± 4.4e6; p = 0.050). Splenic IL-10 was elevated in SLE mice (4.3e7 ± 1.0e7 vs. 8.9e6 ± 8.3e5; p = 0.003), and curcumin treatment did not alter its expression (4.9e7 ± 1.0e7 vs. 4.3e7 ± 1.0e7; p = 0.536) (Figure 3B). Next, we measured pro-inflammatory cytokines in the renal cortex and medulla. Renal cortical TNF-α was elevated in SLE mice compared to controls (8.2e5 ± 3.6e5 vs. 1.0e6 ± 7.9e4; p = 0.010) and curcumin-treatment increased expression in SLE mice (8.2e6 ± 1.6e6 vs. 8.2e5 ± 3.6e5; p = 0.002) (Figure 3C). Renal cortical TGF-β1 was elevated in SLE mice (1.9e5 ± 7.2e4 vs. 7.7e3 ± 2.6e3; p = 0.006) while curcumin-treated SLE mice trended toward increased expression (3.5e5 ± 1.2e5 vs. 1.9e5 ± 7.2e4; p = 0.270) (Figure 3D). Renal medullary TNF-α was increased in SLE mice compared to controls (8.6e6 ± 1.6e6 vs. 1.2e6 ± 7.0e5; p = 0.001) and curcumin treatment increased its expression in SLE mice (2.5e7 ± 4.9e6 vs. 8.6e6 ± 1.6e6; p = 0.005) (Figure 3E). Renal medullary expression of IL-1β was elevated in curcumin-treated SLE mice (3.8e5 ± 1.2e5; p = 0.028) while curcumin-treated SLE mice did not differ in expression of this cytokine (5.6e5 ± 1.8e5 vs. 3.8e5 ± 1.2e5; p = 0.360) (Figure 3F). Lung IL-10 was not elevated in SLE mice (2.1e7 ± 3.0e6 vs. 2.6e7 ± 5.5e6 intensity units; p = 0.825), but did increase in curcumin-treated SLE mice (3.6e7 ± 5.0e6 vs. 2.1e7 ± 3.0e6; p = 0.010) (Figure 3G). Lung TNF-α was elevated in SLE mice (1.3e7 ± 1.3e6 vs. 7.7e6 ± 2.0e5 intensity units; p = 0.004), while curcumin-treatment did not affect its expression (1.1e7 ± 4.2e5 vs. 1.3e7 ± 1.3e6; p = 0.941) (Figure 3H).

Figure 3A

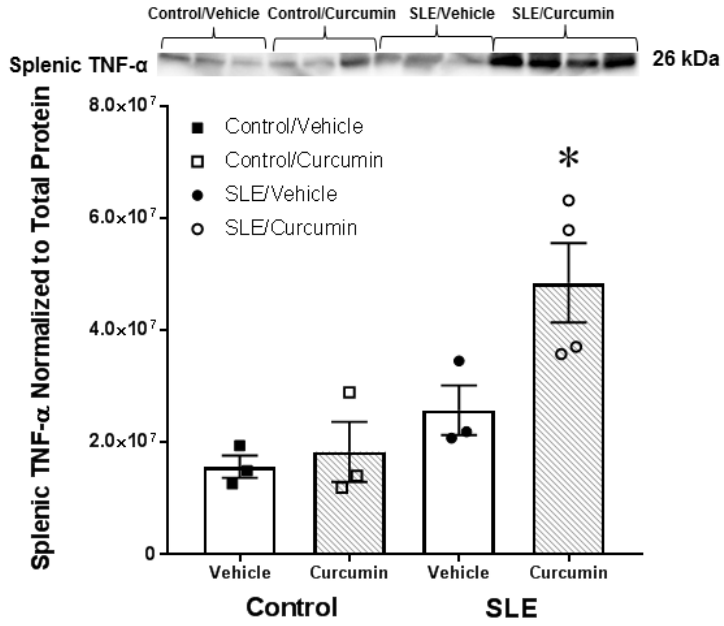


Figure 3B

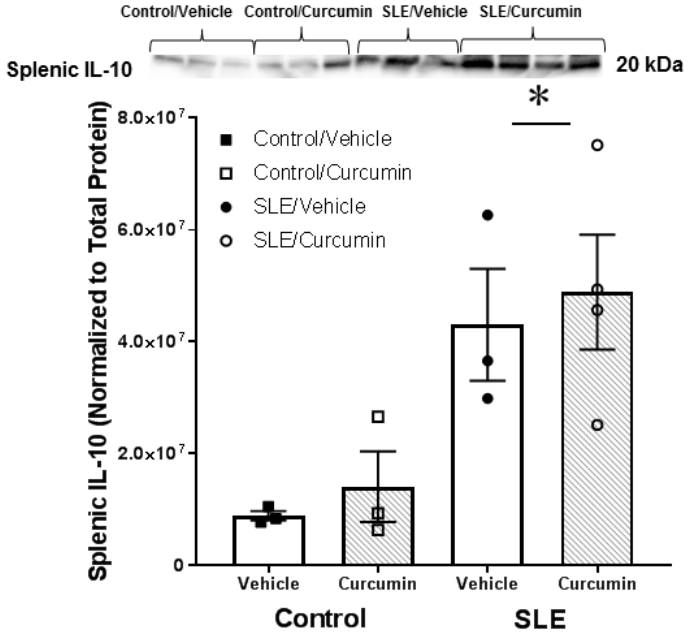


Figure 3C

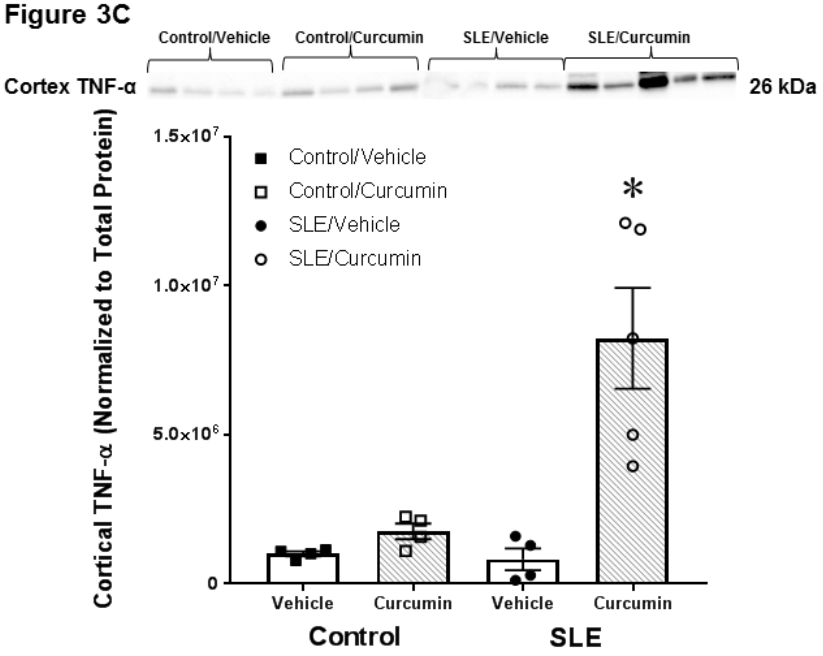


Figure 3D

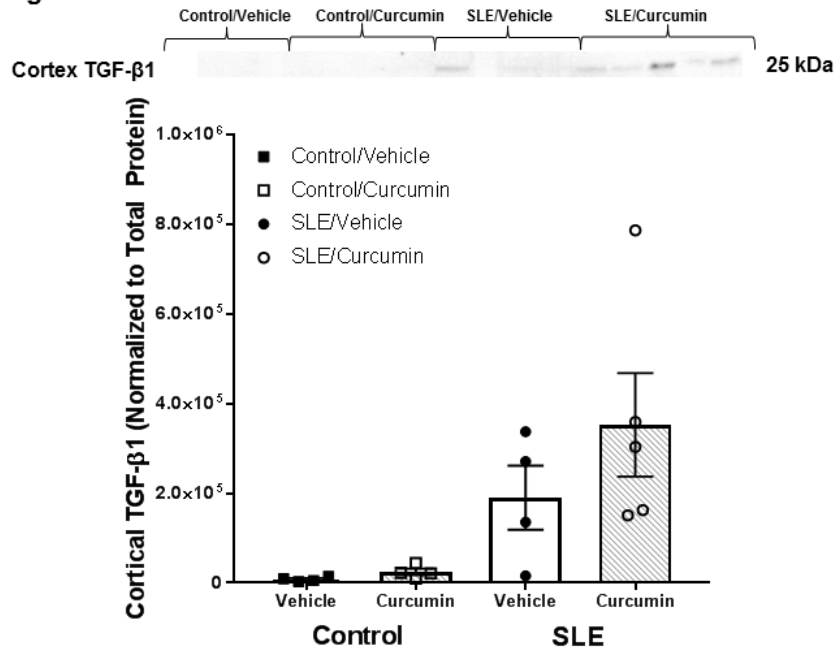


Figure 3E

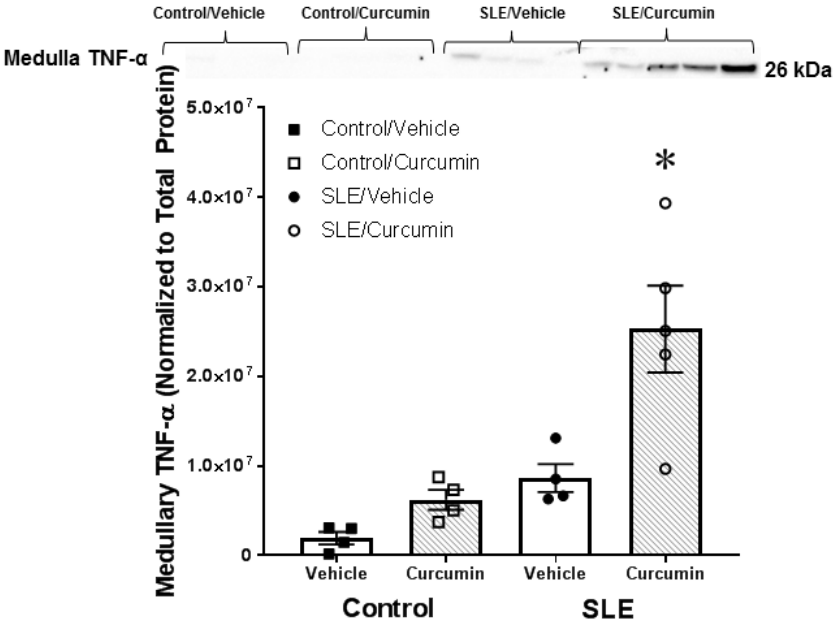


Figure 3F

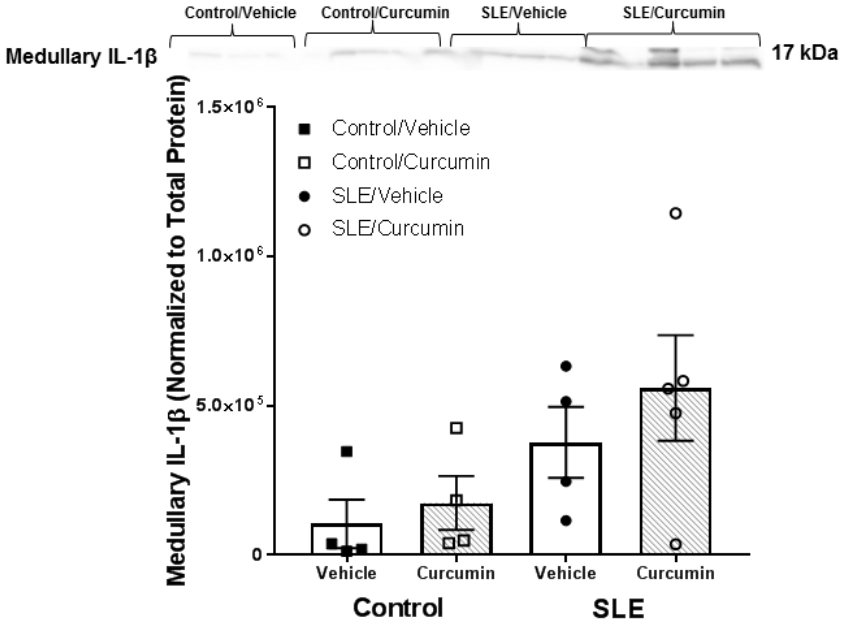


Figure 3G

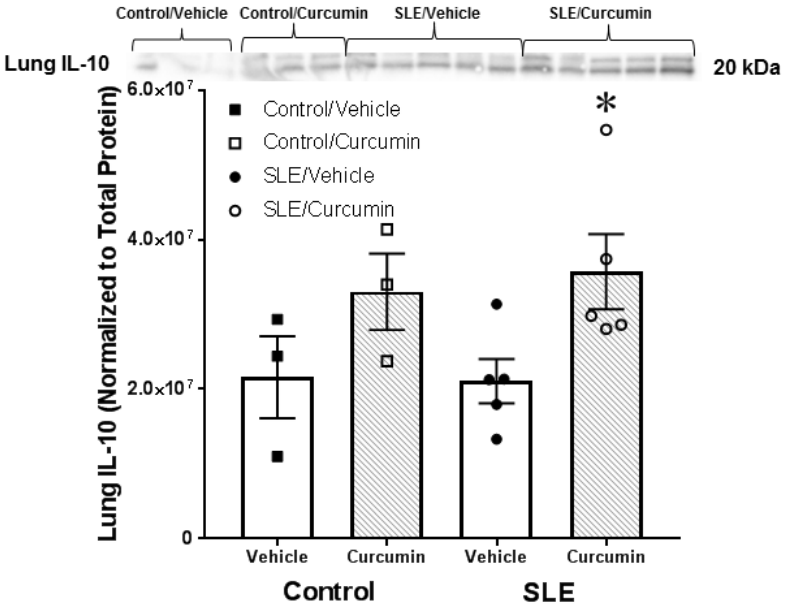


Figure 3H

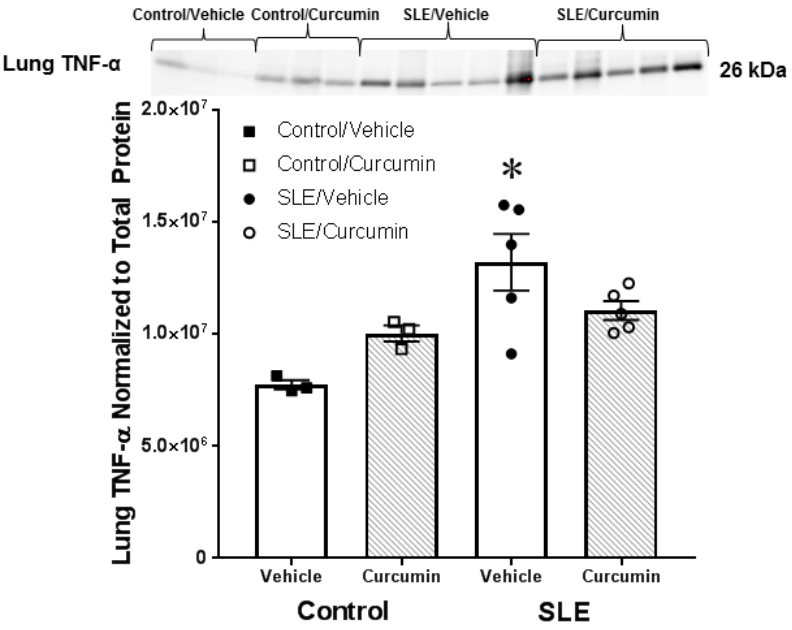


Figure 3. Curcumin increased splenic and renal pro-inflammatory cytokine expression in SLE mice. Values are presented as mean ± SEM. A two-way ANOVA with repeated measures was

used to make statistical comparisons, and p values (all $p < 0.05$) and accompanying symbols were determined using the results of Holm-Sidak post-hoc analysis. n = 4-5/group

Curcumin increases the incidence of albuminuria in SLE mice but does not affect mean arterial pressure in SLE mice. (Figure 4) Curcumin-treated SLE mice developed an albuminuria of > 300 mg/dl at an accelerated rate compared to vehicle-treated SLE mice and controls. SLE mice had a higher mean arterial pressure than control mice (129 ± 4 vs. 114 ± 5 mmHg; p = 0.006). Curcumin treatment did not affect blood pressure in SLE mice, although there was a trend towards an increase (136 ± 4 vs. 129 ± 4 mmHg; p = 0.284). A similar, non-significant increase was also observed in curcumin-treated control mice (120 ± 4 vs. 114 ± 5 mmHg; p = 0.397).

Figure 4A

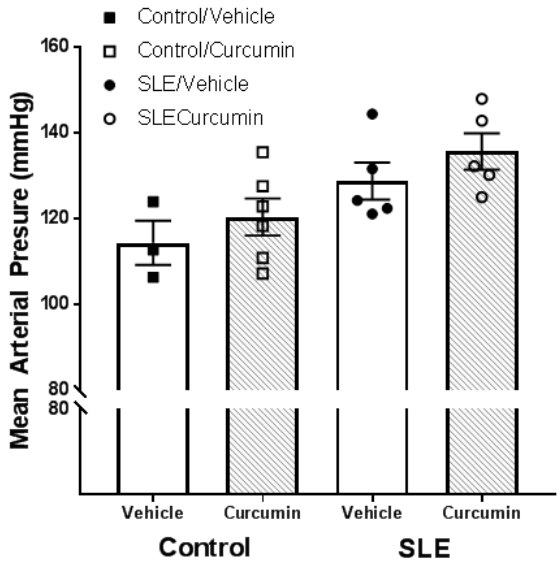


Figure 4B

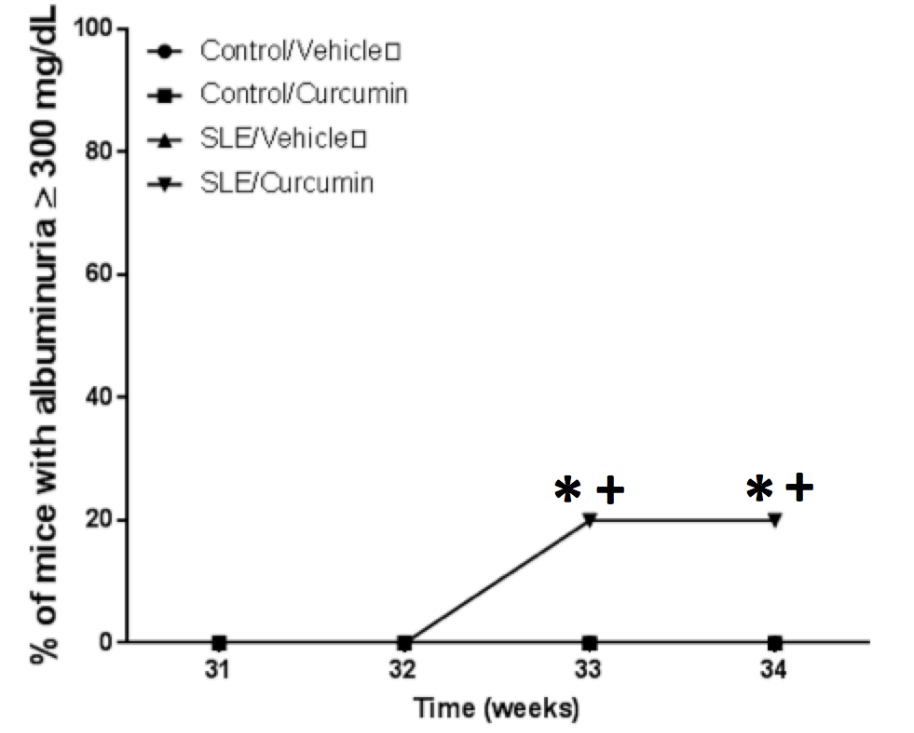


Figure 4. Curcumin did not impact blood pressure and increased the incidence of albuminuria in SLE mice. A. Mean arterial pressure (MAP) was elevated in SLE mice compared to controls. Curcumin did not alter MAP in SLE mice. B. The incidence of albuminuria > 300 mg/dl did not differ between SLE and control mice. Curcumin treatment increased its incidence in SLE mice. Values are presented as mean ± SEM. A two-way ANOVA with repeated measures was used to make statistical comparisons, and p values (all $p < 0.05$) and accompanying symbols were determined using the results of Holm-Sidak post-hoc analysis. n = 4-5/group

DISCUSSION

In this study, we found that chronic intraperitoneal administration of curcumin may have been detrimental to SLE mice, with the findings that 1) intraperitoneal curcumin increased splenic and renal inflammation in SLE mice, 2) increased the prevalence of renal injury in SLE mice, and 3) did not affect blood pressure in SLE mice. One explanation for this is that the intraperitoneal route of administration, in conjunction with the chosen dose, after being repeated for 30 consecutive days, resulted in the curcumin-oil suspension being sequestered in the abdominal area. Intraperitoneal injection was used as an alternative to oral gavage in order to prevent repetitive trauma to the oropharynx and esophagus over the 28-day treatment period, as well as for its increased bioavailability compared to oral administration. Many studies have similarly utilized intraperitoneal injection of curcumin as a mode of administration, but usually not for more than 7 consecutive days and, at most, 20 days. These studies have also employed doses in mice and rats ranging from as low as 5 mg/kg (Zunino et al., 2013), to 50 mg/kg (Avasarala et al., 2013; Sorrenti et al., 2018), 100 mg/kg (Banji, Pinnapureddy, Banji, Kumar, & Reddy, 2011) and 200 mg/kg (L. Liu et al., 2014; Tamaddonfard, Erfanparast, Hamzeh-gooshchi, & Yousofizadeh, 2012). A study utilizing a mouse model of asthma involved administration of curcumin via intraperitoneal injection at a dose of 200 mg/kg daily over a period of 20 days, and did not report increased morbidity nor mortality in the curcumin-treated groups (Oh et al., 2011). It is likely that the dose chosen for intraperitoneal injection in this context was appropriate, but the duration and frequency of the treatment period may have prevented adequate uptake of the curcumin. The sequestration of curcumin in the abdomen may partially explain why the treatment promoted anti-inflammatory effects in the lung, which are located in a separate compartment of the body; the diaphragm completely prevents infiltration of

intraperitoneally administered compounds. However, the kidneys are located in the retroperitoneal space and separated from the anterior abdomen where the curcumin remained. Due to the increase in pro-inflammatory cytokines in the spleen, which is situated in the intraperitoneal region, it is possible that the accumulation of curcumin adjacent to the spleen was treated as a foreign body by spleen lymphocytes, and promoted inflammatory injury of the kidneys. It is also likely that the chronic illness of the SLE mice rendered them especially vulnerable to the accumulation of curcumin. Post-mortem, we observed that intraperitoneal accumulation was much worse in SLE mice, leading to what appeared to be a peripheral foreign body reaction, while there was a less dense abdominal deposit present in the control mice. This outcome was mirrored by a study utilizing a murine model of leukemia in which a 100 mg/kg dose over 7 consecutive days was found to exacerbate mortality in leukemic mice compared to controls, prompting the investigators to construct a dose-response curve and determine that 5 mg/kg was a better-tolerated dose that did not promote mortality (Zunino et al., 2013). This study, in conjunction with the increased mortality of curcumin-treated SLE mice, suggests that mice with chronic disease are not able to tolerate curcumin at the same doses as their healthy counterparts. Ways to optimize curcumin administration in the future include constructing a dose-response curve, as in the murine leukemia study, to determine a more tolerable dose, as well as alternative delivery methods. Others have developed sustained delivery implant techniques and found that polymeric implants grafted into rats elicited foreign body reactions, although curcumin-filled implants elicited less of an inflammatory response compared to vehicle-filled ones (Bansal et al., 2011). In the future, alternative delivery methods of curcumin may be optimized in order to maximize bioavailability of curcumin while preventing adverse reactions.

Initially, in spite of the outcomes following 4 weeks of intraperitoneal treatment, we considered that curcumin may improve the disease course of SLE, but that 4 weeks of treatment was not long enough of a treatment to mediate beneficial effects. To this end, we decided to implement a longer treatment regimen beginning at 20 weeks of age, culminating in a treatment period of 14 weeks. This longer treatment period may have been more detrimental than four weeks, as 14 weeks of treatment aggravated the incidence of albuminuria and led to increased mortality of SLE mice. The effects of 14 weeks of curcumin treatment upon the SLE mice are not known, as the entire experimental group either died prior to catheterization surgery, or did not survive the surgery. The increased mortality resulting from this extended treatment period supports our explanation that the chronic accumulation of intraperitoneal curcumin exhibits a cumulative toxic effect to which SLE mice are especially vulnerable. .

Other commonly used curcumin treatment regimens, such as oral gavage and compounding into food pellets, and intravenous transfusion have been therapeutic in rodent models of SLE. More contemporary methods, such as nanoparticle-conjugated formulations of curcumin, were found to reduce the severity of lupus nephritis in NZM2410 mice (Young et al., 2015) and may comprise an efficacious means of targeted therapy in lupus nephritis patients. However, we are ultimately more concerned with whether curcumin could improve outcomes in human SLE. Curcumin is most commonly employed as an over the counter nutritional supplement, typically in capsule form. Intraperitoneal injection or intravenous infusion of curcumin have not been studied in patients, and their safety and efficacy have not been established in humans. Despite the lack of safety data, naturopathic and alternative medicine practitioners continue to advocate the use of intravenous curcumin infusion. Notably, a 31 year old woman, during a visit to her naturopathic practitioner, went into cardiac arrest minutes after

receiving an intravenous infusion of curcumin, then perished from anoxic brain injury following a prolonged resuscitation attempt (Lasoff et al., 2018). This case, in conjunction with the minority of animal studies that report increased morbidity and mortality from curcumin, call for an increase in rigorous investigation of the potential dangers of unregulated natural remedies.

Studies have been conducted in both human patients with SLE and SLE mouse models. One challenge with interpreting the literature from this disease process is that SLE has a multitude of systemic consequences, and there are at least three different scoring systems to represent the extent of disease severity, including the SLE Disease Activity Index (SLEDAI), which is most commonly used in the US. Additionally, the timing of curcumin administration may play a crucial role in its therapeutic effects in SLE. A study involving 24 patients with relapsing or refractory lupus nephritis demonstrated that chronic administration of oral curcumin capsules decreased proteinuria, hematuria, and systolic blood pressure (Khajehdehi et al., 2012). An *in vitro* study measured the effects of curcumin upon isolated, induced helper T 17 cells (Th17) cells from SLE patients and healthy controls, finding that curcumin added to the media was able to decrease the ratio of Th17 to regulatory T cell (Treg) populations over the course of 72 hours (Handono, Pramata, Endharti, & Kalim, 2015). Another study that supports the proposed therapeutic actions of curcumin upon Treg cells was conducted by Lee et al. utilizing the established New Zealand Black/White F1 (*NZBWF1*) strain, where supplementation at 18 weeks of age until week 32 was able to decrease pro-inflammatory cytokine expression in the kidneys and increase the FoxP3 transcription factor associated with regularly T (Treg) cell development (H. Lee, Kim, Lee, Chung, & Bae, 2013). In all of the mentioned studies, in addition to slowing the progression of renal injury, curcumin also appeared to play a role in decreasing disease severity, as the plasma double stranded-DNA autoantibody titer, a hallmark

finding in SLE, was decreased by curcumin intervention, which indicate that, with the ideal route of administration and dosing, curcumin exerts therapeutic anti-inflammatory effects in both human SLE patients and murine models of SLE. Future studies that identify optimal delivery methods may be indicated to promote the use of curcumin and other natural compounds as alternative or adjunctive treatments for SLE.

SUMMARY

Curcumin has been studied heavily for its antioxidant, anti-inflammatory, and anti-cancer properties, as well as in a plethora of disease models as a supplement or adjunctive treatment. Curcumin may also impact neuro-immune pathways by activating vagal afferents. Notably, curcumin has also been studied in a variety of murine SLE strains, where it has been demonstrated to exhibit anti-inflammatory effects directly upon immune cell populations involved in lupus nephritis. This chapter investigates the effects of chronic intraperitoneal curcumin injection in the setting of lupus hypertension, as well as the possible limitations that may have contributed to the study results. Excessive, repeated dosing (especially when administered intraperitoneally, which yields higher bioavailability than the more conventional oral gavage technique) may have led to increased morbidity and mortality in SLE mice. These limitations and observations illuminate possible precautions that may guide future use of curcumin as a therapeutic agent or supplement in the treatment of SLE.

OVERALL DISCUSSION

As an increasingly prevalent global malady with a multifactorial pathogenesis, hypertension must be investigated with respect to as many potential etiologies as possible, especially chronic inflammation. Furthermore, the presence of autoimmunity in the form of autoantibodies in the setting of essential hypertension (Kristensen & Andersen, 1978) identifies SLE as a unique disease model in which hypertension occurs and must be investigated. Overall, chapters I, II, and III lend support to the hypothesis that dysregulation of neuroimmune pathways is present in an established mouse model of lupus hypertension, and that interventions targeting these pathways may provide more favorable clinical outcomes. Chapter I suggested that afferent vagal sensitivity was comparable in control and SLE mice, which is demonstrated by similar increases in paraventricular neuron activation, but nevertheless shows differing HPA responses to an inflammatory challenge. Future studies may further investigate other sites of central afferent vagal reactivity, such as by quantifying c-Fos responses to LPS challenge in additional brain regions including the dorsal motor nucleus of the vagus and the nucleus ambiguous, as well as identify additional sites of HPA axis dysfunction in SLE, such as the adrenal cortices. In addition, ACTH challenge may reveal a disparity in corticosterone release between control and SLE murine strains. Chapter II indicates that chronic efferent vagus nerve potentiation, mediated by the cholinergic agonist galantamine, reduces blood pressure, renal inflammation, and renal injury in SLE. LPS challenge in the *NZBWF1* mouse model of SLE resulted in greater corticosterone release compared to controls, which may be ultimately insufficient to attenuate the peripheral cytokine release induced by LPS. These sets of findings were anticipated. Future studies may investigate whether other reversible cholinesterase inhibitors have a similarly therapeutic effect in lupus hypertension. Additionally, another pretreatment study that measures

blood pressure after chronic discontinuation of galantamine may provide insight as to whether blood pressure (and renal injury) return to injurious levels once efferent vagus nerve potentiation has ceased. The finding in chapter III that a chronic state of unilateral cervical vagotomy led to reductions in blood pressure and renal inflammation was unanticipated. Existing studies involving chronic vagotomy have not investigated its effects on blood pressure, although the multiple effects of the vagus nerve upon the heart and immune system warrant attention to blood pressure regulation in its absence. It is likely that unilateral vagotomy results in compensation by the intact vagus nerve, though it is unclear why such compensation would lead to increased anti-inflammatory consequences. Future studies may utilize implantable blood pressure telemetry devices in order to measure fluctuations in blood pressure in the weeks following unilateral vagotomy, as well as utilize a subdiaphragmatic vagotomy in an additional subset of mice to evaluate whether all anti-inflammatory effects are abrogated by this selective, total elimination of efferent vagal activity.

There are interesting connections linking the findings from Chapters I, II, and III, which illustrate unique aspects within the inflammatory milieu of SLE mice. As discussed in Chapter I, TLR-4 is a transmembrane protein responsible for the recognition of LPS, and helps mediate innate immune system responses. Additionally, TLR-4 is required for HMGB-1-dependent signaling. Figure 4C in Chapter II, which quantifies HMGB-1 expression in the spleen, shows an augmented concentration of this pro-inflammatory cytokine in SLE mice. The finding that LPS challenge elicits a heightened plasma corticosterone response in SLE mice, in addition to the observation that splenic HMGB-1 expression is augmented in SLE mice, suggests that TLR-4 expression may likewise be increased in SLE mice compared to NZW controls, and that perhaps this may contribute to immune system dysfunction and autoimmunity in SLE mice.

Chapter IV, which focused on intraperitoneal curcumin administration, may have been limited by suboptimal dosing and/or mode of administration, and did not yield results that were applicable to the role of neuroimmune pathways in SLE, but gave way to additional observations that may nevertheless help guide future adjunctive treatments in SLE. The outcomes of SLE mice following both 4 weeks and 14 weeks of intraperitoneal curcumin administration demonstrate that SLE mice are more vulnerable to foreign body reactions, evidenced by worsened renal injury and systemic inflammation in the former, and to increased mortality in the latter. These results were unanticipated, as the vast majority of the curcumin literature reports either negligible or beneficial outcomes in conditions ranging from cancer to asthma. The finding that intraperitoneal curcumin was injurious to SLE mice in our study may have stemmed from the 28 consecutive, daily injections, employed in the treatment period, in addition to the chronically ill SLE mice being less able to tolerate this compound. As a whole, these results contribute to a small body of studies where excessive curcumin dosing was found to increase mortality in murine models of chronic disease (Zunino et al., 2013), and shed insight as to the tolerability of this compound between healthy and diseased cohorts. Future studies may compare oral gavage and other alternative delivery methods to intraperitoneal injection. Nanoparticle formulations that could specifically target afferent vagal neurons in the gut may especially promising in promoting endogenous neuroimmune pathway activity.

There are certain limitations that should be considered in light of these studies. The *NZBWF1* model presents with a highly variable phenotype wherein several animals in one cohort may be predisposed toward fulminant renal failure at a much earlier time point than its counterparts (unpublished observations). The SLE phenotype notably varied, with certain mice experiencing fulminant renal failure compared to others in their cohort. Certain mice that met

initial inclusion criteria may experience fulminant SLE flares unpredictably throughout chronic treatment protocols, resulting in unanticipated deaths. Blood clots also happened following carotid catheterization surgery, leading to strokes in certain animals; these animals were necessarily excluded from studies due to effects of cerebrovascular injury upon blood pressure regulation, as well as ischemic brain and end-organ injury leading to inflammatory aberrations. Additionally, smaller blood clots may have affected other mice without the sequelae of strokes or dramatic losses of function, thereby going undetected while mediating end organ injury. However, the variable disease phenotypes of SLE mice is similar to how SLE presents in an unpredictable and variable fashion in human patients (Barr, Zonana-Nacach, Magder, & Petri, 1999; Merrell & Shulman, 1955). Another limitation may entail the use of single, final blood pressure measurements at the end of treatment periods instead of time course blood pressure data from baseline throughout treatment also do not shed light on the transient changes of blood pressure throughout the courses of the studies, although likewise, there are only final measures of inflammation. A solution for this is to utilize implantable telemetry devices to continuously monitor blood pressure throughout the baseline, treatment, and end of the study. Additionally, serial blood sampling throughout the study could be performed in order to assess fluctuations in pro-inflammatory cytokine circulation. In addition to the spleen, it may be enlightening to measure the effects of the neuro-immune interventions carried out in these studies amongst other lymphoid tissues, such as the thymus, bone marrow, or lymph nodes, which possess adrenergic innervation (Bachmann et al., 2019; Lubahn et al., 2014; Nakai, Hayano, Furuta, Noda, & Suzuki, 2014). Lastly, the studies in Chapters II-IV utilized a prophylactic treatment prior to the development of renal injury in SLE mice, as opposed to investigating the effects of an intervention in the context of established disease. This is significant in light of the fact that most

SLE patients unexpectedly develop SLE and may not receive a diagnosis until later stages the disease. A solution to this limitation would be to repeat the interventions at an advanced time point, such as at 34 weeks of age, or to only include mice that had significant albuminuria.

Additionally, genetic mouse models of lupus do not encapsulate the full spectrum of human SLE, with each strain encompassing several manifestations of the disease. The choice to utilize the *NZBWF1* strain is a result of this particular strain reliably developing hypertension at a certain stage of life, in addition to the characteristic anti-dsDNA autoantibodies whose titer correlates with disease severity. It may be worthwhile to investigate neuroimmune interventions in other genetic strains of murine lupus, as this would provide insight as to the degree of neuroimmune aberration and its contributions to other pathologic sequelae of SLE. MRL/lpr mice are another well-established murine strain of SLE that are unique in that they reliably express the full spectrum of autoantibodies seen in SLE patients, including anti-dsDNA, anti-ANA, anti-Sm, anti-Ro, and anti-La (W. Li, Titov, & Morel, 2017). The NZM2410 strain is frequently utilized to study lupus nephritis (Young et al., 2015). BALB/c mice, when injected with the hydrocarbon pristane, develop anti-Su, anti-U1RNP, anti-U2RNP, and anti-Sm autoantibodies, in addition to a lupus phenotype (Satoh & Reeves, 1994). While there are induced SLE models created by hydrocarbon exposure to wild type rodents, these may not develop hypertension and other lupus manifestations to the same extent as genetic models (Richard & Gilkeson, 2018), although they may yield additional insights into environmental factors that contribute to the development of SLE. It would be worthwhile to determine whether existing genetic murine models of SLE also exhibit hypertension throughout their lifetime, or to develop additional mouse models of lupus hypertension, so as to reflect the genetic and racial diversity observed in various human lupus patient populations worldwide.

OVERALL CONCLUSIONS

Hypertension continues to grow in scope and breadth as a global health crisis, both in new cases in essential hypertension, as well as in the increasing prevalence of treatment-resistant hypertension. The interplay between the nervous and immune systems and its role in mediating chronic inflammation leading toward hypertension inspired this inquiry into whether endogenous neuroimmune pathway aberration is present in SLE, an autoimmune disease that frequently presents with hypertension, and whether interventions targeting these pathways may improve clinical parameters of SLE. In summary, several of the most important findings were that (1) the corticosterone response to LPS challenge may be inadequate in mice with SLE despite comparable afferent vagal sensitivity; (2) increased efferent vagus nerve activity mediated by central cholinergic potentiation led to reduced blood pressure, renal inflammation, and renal injury in lupus hypertension; (3) unilateral cervical vagotomy reduces blood pressure and renal inflammation, and; 4) curcumin sequestration in the abdomen is injurious to SLE mice, indicating that afferent vagal neurons in the gastrointestinal tract may mediate anti-inflammatory effects of orally ingested curcumin.

Lastly, by no means is this dissertation exhaustive in exploring the role that these two neuro-immune pathways have in SLE, nor of other neuro-immune pathways not investigated in these studies. The existing body of research into HPA axis dysfunction in SLE (and other rheumatic diseases) leaves many questions unanswered, particularly as to what components of the HPA axis tend to exhibit dysfunction and how these aberrations may change over the course of a lifetime. The scope of this hypothesis, though ambitious for a dissertation, have emphasized less commonly explored foci of inquiry, and may lead to future research that more comprehensively delineates the involvement of neuroimmune mechanisms in autoimmunity.

Human studies and potentially clinical trials investigating the use of vagus nerve-targeted interventions in patients with documented lupus hypertension may be instrumental in assessing the clinical impact of targeting neuroimmune pathways and the resulting effects on blood pressure. Additional preclinical studies focused on other neuroimmune pathways that may be involved in lupus hypertension are also warranted. Such investigations may promote creativity and new ways of conceiving the multifocal pathogenesis that underlies both SLE and hypertension.

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