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Systemic lupus erythematosus (SLE) is a female-dominant autoimmune disease associated with hypertension. We confirmed that SLE develops later in life in male vs. female SLE mice (35 vs. <30 weeks), yet both sexes develop hypertension by 35 weeks. Renal injury is a factor in hypertensive female SLE mice only, so we aimed to investigate this latent sex difference. We hypothesized that increased toll-like receptor 7 (TLR7), an immune mediator that instigates tissue damage, promotes renal injury in female SLE mice. We found that renal cortical expression of TLR7 was indeed higher in female SLE mice. In a follow-up study we found that renal hemodynamics were impaired in female SLE mice, but not males. Our data suggest that while the hypertension in female SLE mice may be due to renal mechanisms, hypertension in males is not. Future studies will dissect sex-specific factors that should be considered when treating hypertensive patients with underlying autoimmunity.

SEX DIFFERENCES IN TOLL LIKE RECEPTOR 7-MEDIATED

RENAL INJURY IN A MURINE MODEL OF

AUTOIMMUNE-INDUCED

HYPERTENSION

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SEX DIFFERENCES IN TOLL LIKE RECEPTOR 7-MEDIATED RENAL INJURY IN A MURINE MODEL OF AUTOIMMUNE-INDUCED HYPERTENSION

INTERNSHIP PRACTICUM REPORT

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INTRODUCTION

Cardiovascular disease is the leading cause of death in the US and throughout the developed world. Hypertension is a major risk factor for cardiovascular diseases including myocardial infarction, heart failure and stroke [1]. The etiology of hypertension is multifactorial, and the wide range of current therapeutic options reflects the diversity of the disease. Although the associated healthcare costs exceed $130 billion in the US alone, current anti-hypertensive medications only help control hypertension in approximately 54% of adults in the US [2, 3]. There is still a large population of patients with hypertension resistant to traditional courses of treatment. This suggests that the etiology of hypertension may be different within this contingent and emphasizes the need to study novel mechanisms contributing to the development of resistant hypertension.

There is increasing evidence to support autoimmunity as a contributing factor to hypertension due to the actions of autoantibodies that can induce inflammatory responses [4, 5]. Additionally, many studies have shown the contribution of immune cells like T-cells [6] and B cells [7, 8] to hypertension. Indeed, the prevalence of hypertension is increased in patients with autoimmune conditions [9] when compared to the general population. Interestingly, the prevalence of resistant hypertension is nearly doubled in some autoimmune populations [10], which may indicate that the actions of traditional hypertension therapeutics are less effective in patient populations with autoimmune-induced hypertension. Conversely, in diseases such as HIV in which the immune system is compromised, patients are less likely to be hypertensive [11]. More

research is needed to understand the roles of autoimmunity, overactive T and B cells, and the presence of autoantibodies in the pathogenesis of hypertension.

SLE is a chronic inflammatory disorder and classic example of autoimmunity. The disease is characterized by a overactivation of T and B cells that causes elevated plasma levels of anti-nuclear autoantibodies (e.g. anti-double-stranded DNA (dsDNA) autoantibodies). These autoantibodies promote the formation of immune complexes that embed themselves into tissues. The resulting inflammation causes damage notably in the kidneys, but also in the skin, musculoskeletal system, and cardiovascular system [12]. There is a high prevalence of hypertension in SLE patients, and this is associated with cardiovascular disease, the leading cause of mortality in this patient population [13]. Accordingly, we use SLE as a disease model to determine how autoimmunity contributes to hypertension.

There are clear sex differences in the prevalence of the disease. SLE is a female-dominant disease, with nearly 90% of all patients being young women of child-bearing age. The female bias in SLE has not been completely described, although X chromosome dosage, sex hormones and genetic risk load seem contribute to the phenomenon. Increased X-chromosome dosage due to incomplete inactivation is thought to result in the increased expression of genes that confer risk to SLE. This notion is particularly supported by Klinefelter's males who carry the XXY genotype and have a 14 times higher risk of developing SLE when compared to XY genotype males [14]. Additionally, sex hormones were thought to play an immediate role in the female predisposition to SLE, with estrogen playing a permissive role in the development of the disease. However, recent studies using animal models view the contribution of estrogen to SLE as complex, with the

hormone alternating between a protective and pathogenic role at different points in life [15]. Recent studies involving the relative genetic risk load for SLE in men and women have produced conflicting results, with some groups concluding men required a higher genetic susceptibility to develop the disease, while others noticed no sex difference in the necessary risk load [16, 17]. While the notable female bias is still being characterized, sex differences in the development of the disease itself are even less defined. According to cohort studies, SLE in men is diagnosed at a later age and associated with higher prevalence of organ damage and cardiovascular manifestations (myocardial infarction, peripheral vascular disease) [18]. However, it is not clear whether these outcomes are due to the progression of the disease or because male patients do not seek medical expertise as often or as soon as symptoms present, which itself could lead to delayed intervention and more severe health consequences [19, 20]. Regardless, the underlying mechanisms behind these apparent sex differences in the pathogenesis of SLE are not clearly understood.

We have traditionally utilized a well-established mouse model of spontaneous SLE, female *NZBWF1* mice, to investigate immune mechanisms involved in the development and maintenance of hypertension in the setting of chronic inflammation. The *NZBWF1* strain is useful in studying SLE as the heterogenous nature of the disease in humans is reflected in the genetic construction of these mice. This strain is the first generation of a cross between the New Zealand White (*NZW*) and New Zealand Black (*NZB*). While neither parental strain develops overt symptoms of SLE, both exhibit mild autoimmunity, indicated by elevated levels of anti-nuclear antibodies (ANA) [21]. Female *NZBWF1* exhibit significantly higher levels ANA levels and develop immune-complex mediated glomerulonephritis[22]. The pathogenesis of SLE in humans is

marked by the variable involvement of multiple systems. Investigations into genes that contribute to SLE susceptibility in *NZBWF1* have mapped certain phenotypes to particular loci. Contribution of loci can contribute to one or multiple pathologies in the disease, indicating the SLE-like symptoms in *NZBWF1* results from the additive contributions of multiple variants from the NXB x NZW cross that individually do not confer the full pathology of SLE [23]. A study by Kono et. al identified several loci that conferred susceptibility to immune-complex mediated glomerulonephritis, anti-chromatin autoantibody production, and mortality. Of the loci explored, only the major histocompatibility complex was linked to all outcomes. Mortality at 12-months was greatest in mice with a heterozygous genotype at the MHC locus, perhaps indicating a interaction between the two haplotypes contributes to a more severe phenotype than a homozygous pairing [24].

We have consistently found that as the disease progresses in female SLE mice, dsDNA autoantibodies develop, form complexes and deposit into the kidney, causing lupus nephritis. The resultant renal inflammation, results in hypertension by the time the animals reach of age [25-28]. Our central hypothesis is that male SLE mice would have a similar disease progression as the females, but perhaps shifted to later in life. Our preliminary data also indicate that male SLE mice do not develop renal injury as early as the female SLE mice (**Figure 2**), yet both male and female SLE mice are hypertensive by 35 weeks of age (**Figure 3**). This implies that hypertension in male SLE mice may arise through an alternative mechanism. Further, this may indicate that the development of autoimmune-associated hypertension, and potentially resistant hypertension, could occur through different pathways in males and females.

Toll-like receptors (TLRs) are immune receptors that recognize specific microbial elements and subsequently activate the innate immune response [29]. TLRs responsible for sensing nucleic material are bound to the membrane of the endosomes of immune cells [30]. Toll-like receptor 7 (TLR7) is one such endosomal immune receptor classically activated by single-stranded RNA, but also immune complexes [31]. TLR7 activation eventually causes the production and release of interferon (IFN)-α. IFN-α, normally functions in the innate immune system to protect against viral and bacterial infection; however, the type 1 IFN signature has been implicated in many autoimmune disorders (e.g. SLE, rheumatoid arthritis) as a driver of apoptotic factor and inflammatory cytokine production [31]. In SLE, IFN-α specifically enhances B-cell proliferation which promotes increased production of autoantibodies responsible for much of the renal injury and tissue damage seen in the disease [31-33].

The association between TLR7 activation and SLE has been previously examined in murine models, where gene duplication or overexpression of the region encoding TLR7 promotes the development of SLE-like symptoms. Additionally, both *Tlr7* in mice and *TLR7* in humans are found on the X-chromosome, and incomplete X-chromosome inactivation may result in increased dosage of this gene [34]. **Based on this, we hypothesized that increased sensitivity and expression of TLR7 in female SLE mice plays a role in renal injury not seen in males, and that hypertension in male SLE mice may arise from an alternate pathway (e.g., impaired renal hemodynamics).**

The aim of the current study is to investigate renal TLR7 as a potential contributor to the latent sex difference seen in the development of hypertension in male and female SLE mice. While an

increased sensitivity and expression of TLR7 in SLE females would help explain the increased susceptibility to renal injury in women with SLE, reduced TLR7 expression may also provide a reason why we did not see renal injury in male SLE mice. We anticipated that female SLE mice would display both an increased expression and sensitivity of TLR7, yielding higher production of IFN-α.

The information gained from this project would benefit the lupus community as recent clinical trials for therapies designed to treat SLE target the TLR7-IFN-α pathway. If our hypothesis is correct, the effectiveness of these drugs may be different in males and females, as the TLR7 target may not be as responsible for the symptoms of SLE in male patients. Additionally, this project affords additional insight into the contributions of autoimmunity to resistant hypertension and if sex should be considered in treatment plans.

RESEARCH DESIGN AND METHODOLOGY

Animals

This project used female and male SLE (*NZBWF1*) and control (*NZW/LacJ*) mice from Jackson Laboratories (Bar Harbor, ME). The *NZBWF1* strain is a spontaneous mouse model of lupus that has been used extensively since the 1960s [35]. Mice in our studies were obtained at 4-6 weeks of age and housed in a temperature-controlled facility with free access to food and water. Body weights were measured weekly starting at 30 weeks of age. Tissues were collected after euthanizing animals at 35 weeks of age. Measurements for this study were conducted at either an "early" or "late" time point and will referenced throughout this document as such. Our early time point ranged from 28-31 weeks of age and the late measurements were taken from 34-35 weeks of age. 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.

Urinalysis and Albuminuria

Animals were placed in metabolic cages for 12-24 hours from 30 through 35 weeks of age. Urine was retrieved from the cage and analyzed via dipstick (Siemens). Each dipstick was placed in a respective container of urine and allowed to develop for 60 seconds. Afterwards, the concentration of albumin in each sample was assessed using the provided colorimetric scale. Samples with a concentration of albumin of 300 mg/dL were scored as positive for albuminuria.

Blood Pressure Measurements

Blood pressure was measured in conscious mice using a catheter placed in the left carotid artery. Mice were anesthetized using isoflurane. An incision was made along the neckline and the left carotid artery isolated. After occluding blood flow, a small incision was made in the vessel using a needle after which a catheter filled with 1% heparinized saline was advanced to the arch of the aorta. The proximal end of the catheter was fixed in place, and the distal end exteriorized by tunneling it subcutaneously around the neck of the mouse and exposing it via an incision made on the posterior side of the neckline. After a one-day recovery period, and a 1-hour acclimation period, blood pressure measurements were recorded for 30 minutes using PowerLab software. This procedure was repeated the next day, after which all mice were euthanized, and tissues collected.

Renal Hemodynamics

Catheters were inserted as described above into anesthetized mice, without exteriorization. Following a posterior incision, the right renal artery was carefully isolated from the corresponding vein and placed in a Transonics (Ithaca, NY) flow probe. Renal blood flow (mL/min) and anesthetized blood pressure measurements (mmHg) were gathered simultaneously for 30 minutes following a 30-minute stabilization period using Powerlab software. Renal vascular resistance (RVR; $mmHg \cdot min \cdot kg \cdot mL^{-1}$) was calculated by dividing mean arterial pressure by renal blood flow normalized to body weight.

Plasma dsDNA Autoantibody Detection

Plasma levels of anti-double stranded DNA (dsDNA) autoantibodies was measured at early and late time points via ELISA (AlphaDiagnostic, San Antonio, TX, USA) per the manufacturer's instructions. Briefly, samples, blanks and controls were added to the appropriate wells in duplicate and incubated for one hour at room temperature. After washing 4 times, anti-mouse IgG HRP was added to each well and incubated for 30 minutes at room temperature. Following another wash cycle, TMB substrate was added to each well, and the plate incubated un the dark for 15 minutes, after which stop solution was also added to the wells. Absorbance at 450 nm was read on a plate reader, yielding an optical density (OD) for each well. Interpretation of results was done through a positive index. The mean concentration (U/mL) of a group of naïve, female 35 week old C57 mice was calculated. The average plus the standard deviation of the group was used to remove outliers from within the control group. The average plus 2 times the standard deviation was divided by each experimental sample concentration to yield their positive index, the threshold for a positive indication of SLE. Values greater than one were positive and values below one were marked negative for SLE.

Measurement of the Renal Expression of Inflammatory Mediators

Renal cortical and medullary TLR7 and TNF-α were examined via Western blot using a stain-free method. Tissues were homogenized in 8 times their weight of RIPA buffer with a protease inhibitor cocktail. Samples were loaded into gels and the samples were subjected to electrophoresis at 250 V for 25 minutes. The gels were then activated using a ChemiDoc (Biorad, Hercules, CA) after which proteins were transferred to a nitrocellulose membrane using a

TurboBlot transfer system (Biorad, Hercules, CA). After confirming protein transfer, the membrane was blocked in 20% Blotto and then incubated with the primary antibody overnight (about 17 hours) at 4 C. After removing the primary antibody solution, the membrane was washed in TTBS. Following washing, the secondary antibody solution was applied to the membrane and incubated for one hour at room temperature. After removing the membrane from the secondary antibody solution, the membrane was imaged before applying Clarity ECL chemiluminescent substrate (Biorad, Hercules, CA) for 5 minutes. Blots were then exposed on a ChemiDoc and resulting images were analyzed using ImageLab software. The pre-ECL image was compared to the exposed image and bands of interest were normalized to total lane protein as previously reported from our lab [25-28, 36]. TLR7 was detected by probing with an antibody against murine TLR7 (1:1000, R&D Systems, Minneapolis, MN), followed by use of an HRP-conjugated donkey anti-rat IgG secondary antibody (1:10000, Rockland, Limerick, PA). TNF-α was detected in a similar manner with use of an anti-murine TNF-α antibody (1:250, Santa Cruz Biotech, Dallas, TX) and a donkey anti-mouse IgG secondary antibody (1:10000, Rockland, Limerick, PA).

Statistical Analysis

Statistical analysis was achieved using SigmaPlot 11.0 (Systat, Richmond, CA, USA). Data are calculated as mean ± standard error of the mean. When appropriate, a two-way ANOVA with or without repeated measures, followed by a Holm-Sidak post-hoc test was used to determine differences between groups, with a $p < 0.05$ indicating a significant difference. A t-test was used to determine differences between male and female SLE mice renal vascular resistance. Based on

a power analysis utilizing data from previous studies using the *NZBWF1* and *NZW* strains, an *n* of 6-8 per experimental group is necessary to achieve statistical significance. All of our data exceed this requirement.

RESULTS

Both Female and Male SLE Mice Produce Autoantibodies Over Time

Plasma levels of anti-dsDNA autoantibodies, a clinical marker of SLE, were present in female SLE mice (6.1e5 ± 9.7e4 activity units) and male SLE mice (2.1e5 ± 4.2e4 activity units) at the earlier time point (**Figure 1**). Because the two-way ANOVA did not reach significance as it pertains to the interaction, we were unable to compare the groups with each other or to female (5.8e4 ± 1.0e4 activity units) and male controls (4.3e4 ± 5.0e3 activity units)).

There were no significant changes in plasma anti-dsDNA autoantibodies from the early time point to the later time point. However, the prevalence of animals that reached the threshold for plasma dsDNA autoantibodies increased from 52% to 60% in SLE male mice and 90% to 97% in SLE female mice. Plasma levels of anti-dsDNA autoantibodies were present in female SLE mice (6.3e5 ± 1.1e5 activity units) and male SLE mice (2.5e5 ± 4.6e4 activity units) at the late time point (**Figure 1**).

Male SLE Mice Do Not Develop Albuminuria and Renal Injury by 35 weeks

Female SLE mice progressively developed albuminuria, an indication of renal injury, with a prevalence of 50% (13 out of 26) by 35 weeks. 4% (1 of 25) of SLE males, none of the male control mice and only 4% (1 of 27) of female controls and developed albuminuria throughout the course of the study (**Figure 2**).

Both Male and Female SLE Mice Are Hypertensive

Due to a lack of significant interaction, no statistical differences can be reported in mean arterial pressure between control and SLE, male and female mice (**Figure 3A**). However if comparing only control female mice, our historical control, to SLE male and female mice, mean arterial pressure was significantly elevated in both male and female SLE mice (**Figure 3B**; males: 158 ± 8, $p = 0.003$; females: 149 ± 5, $p = 0.014$) compared to control females (125 ± 4 mmHg).

Renal TLR7 Expression is Higher in Female SLE Mice

Renal cortical expression of TLR7 was increased in female SLE mice at 35 weeks of age (**Figure 4A**) when compared to both male SLE mice or female controls. Female SLE mice expressed a significantly higher renal cortical expression of TLR 7 (3.9e5 ± 8.0e4 intensity units; normalized to total protein) than both male SLE (7.0e4 ± 1.5e4 intensity units; $p < 0.001$) and female control mice (7.5e4 ± 2.6e3 intensity units; $p < 0.001$). This trend was also true of renal medullary TLR7 (**Figure 4B**) with female SLE mice again expressing significantly higher levels of TLR7 (6.0e5 ± 1.2e5 intensity units; normalized to total protein) than male SLE (1.5e5 ± 4.0e4 intensity units; $p < 0.001$) and female control mice (6.8e4 ± 1.7e4 intensity units; $p < 0.001$).

Renal TNF-α Expression is Higher in Female SLE Mice

Renal cortical expression of TNF-α was increased in female SLE mice (1.3e6 ± 3.7e5; normalized to total protein) at 35 weeks of age (**Figure 5A**) when compared to both male SLE mice (5.1e5 ± 1.6e5 intensity units; $p < 0.001$) and female control (1.2e5 ± 3.4e4 intensity units; $p < 0.001$) mice. This trend was also true of renal medullary TNF-α (**Figure 5B**) with female SLE mice again

expressing significantly higher levels of TNF-α (1.1e7 ± 1.6e6 intensity units; normalized to total protein) than male SLE (2.2e6 ± 6.0e5 intensity units; $p < 0.001$) and female control mice (1.0e6 ± 3.1e5 intensity units; $p < 0.001$).

Renal Vascular Resistance is Higher in Female SLE Mice

Anesthetized mean arterial pressure (male SLE mice = 77 ± 4 mmHg; female SLE mice 89 ± 6 mmHg) was divided by renal blood flow normalized to body weight (male SLE mice = 15.7 ± 1.35 $ml \cdot min^{-1} \cdot kg^{-1}$; female SLE mice 10.6 ± 2.18 $ml \cdot min^{-1} \cdot kg^{-1}$) to yield renal vascular resistance. Female SLE mice had significantly higher renal vascular resistance adjusted for body weight (**Figure 6**) than male SLE mice (10.07 ± 1.23 $mmHg \cdot min \cdot kg \cdot mL^{-1}$ vs. 5.15 ± 0.60 $mmHg \cdot min \cdot kg \cdot mL^{-1}$; $p= 0.008$).

DISCUSSION

This study investigated TLR7 expression as a potential contributor to the renal injury seen in solely female SLE mice. Our hypothesis that female SLE mice would exhibit higher expression of renal TLR7 was based on studies indicating that TLR7 activation by endogenous ligands may be responsible for some of the symptoms seen in SLE, specifically renal damage. Alternatively, we considered higher renal vascular resistance in male SLE mice as possible driver of the hypertension in lieu of any renal injury. The results of this study indicate that both (a) renal TLR7 expression and (b) renal vascular resistance is higher in female SLE mice than male SLE mice. These data suggest a lack of renal involvement in the genesis of hypertension in male SLE mice which may, in part, be due to the comparatively lower expression of TLR7 in these animals.

The higher expression of TLR7 in female SLE mice was consistent with our hypothesis and the supports findings of previous studies asserting that female predisposition to SLE could be linked to increased dosage of TLR7 due to incomplete X-inactivation [37, 38]. The proposition that an increased dosage of the TLR7 gene is linked to heightened susceptibility to autoimmune disorders is strengthened by other mouse models of SLE. Male *BXSB*, another strain of lupus mouse, have a duplication and translocation of the region of the X-chromosome that contains the TLR7 locus and these animals exhibit symptoms of SLE earlier than their female counterparts. While the results of our study do not directly investigate and quantify the gene dosage of TLR7 in female SLE mice, the expression of TLR7 in these animals was shown to be higher than both male SLE and female controls, indicating a correlation between increased TLR7 expression and renal injury in the context of SLE. Activation of TLR7 by various self-antigens, such as single-stranded RNA or

immune complexes, leads to induction of IFN-α which, amongst its many downstream effects, results in heightened antibody release by B cells and increased production of pro-inflammatory cytokines such as TNF-α [32]. Accordingly, we demonstrated lower levels of TNF-α in male SLE mice, possibly as a result of the lower expression and sensitivity of TLR7 in male SLE mice. Chronic renal inflammation can lead to renal injury, so the lower TNF-α may also be linked to the nonexistent renal injury in male SLE mice.

Previous studies in non-autoimmune populations have shown TLR7 to induce comparatively higher levels of IFN-α in females than in males [39]. If this pattern is the same in SLE, it would provide additional support for the notion that the increased incidence of SLE in females is in part due to a difference in the expression and function of TLR7 [38]. While this project confirmed a higher level of renal TLR7 expression in female SLE mice, these data do not quantify the sensitivity of the receptor. The next stages of this project would include measuring levels of renal IFN-alpha expression through RT-PCR in order to determine a sex difference in the response of TLR7. Additionally, while renal TLR7 expression was the focus of the current study, it would be prudent to examine if this pattern is true in other tissue beds. Mice used in this study were followed over the course of 30-35 weeks of age, with tissue samples collected from the animals at 35 weeks. While this design afforded time to measure the progression of renal injury, the impact of age on renal TLR7 expression and other outcome variables was not considered and would provide useful data for future investigations into the development of SLE and its symptoms.

The results of this study provide a possible mechanism for the renal injury seen exclusively in female SLE. The observational design of this study limits the ability to make a causative determination between activation of TLR7 and hypertension. Administering a TLR7 antagonist (e.g. chloroquine, hydroxychloroquine) in male and female SLE mice might alleviate renal inflammation in SLE but may also attenuate the subsequent development of hypertension due to renal injury induced by chronic inflammation. While past drugs targeting TLR7 have been used as a potential treatment for SLE [40, 41], the use of these drugs have not been examined within a context of mitigating eventual cardiovascular outcomes in addition to the "normal" symptoms of SLE such as erythematic rash and pain [42]. Investigating the effects of a TLR7 antagonist would afford the opportunity to further examine the link between autoimmunity, TLR7 and hypertension. An attenuation in the development of hypertension in these animals due to blockade of TLR7 would provide evidence to support TLR7 as a target for the hypertension and increased mortality due to cardiovascular disease seen in SLE.

While the results of this study offer insight into how renal damage is circumvented in male SLE mice, it does not account for the marked development of hypertension in lieu of renal injury. Considering altered renal hemodynamics in male SLE mice as a potential explanation was a first step in teasing out the mechanism behind this phenomenon, as high renal vascular resistance can promote pro-hypertensive pathways such as renin release [43]. However, the observation that female SLE mice had significantly higher renal vascular resistance implies that hypertension in male SLE mice occurs through means yet to be understood.

A potential explanation for hypertension in lieu of renal injury seen in the male SLE mice is autonomic imbalance. Hogarth et. al observed both hyper- and normotensive males had higher muscle sympathetic activity (mSNA) than females [44]. Korobka et al. also noticed a shift in autonomic balance based on sex, albeit a different one, as both hyper- and normotensive women displayed a shift towards parasympathetic dominance [45]. These findings would suggest a sex difference in the balance between sympathetic and parasympathetic tone in males and females. Sex differences have also been observed in autonomic regulation of hypertension, with Hart et. al observing that mSNA is correlated with increased total peripheral resistance in men but not women [46]. The increased sensitivity to mSNA in males may also extend to renal sympathetic nerve activity (rSNA), which has been shown to play a role in the development of hypertension, fluid retention and other cardiovascular diseases [47]. Previous studies by our group have linked impaired vagal tone to dysfunction in anti-inflammatory mechanisms in female SLE mice [36], which contributes to the renal damage seen in the disease. This parasympathetic dysfunction may occur in both sexes, but the SLE males might prove to be more responsive to the autonomic imbalance. Taken together, there is a possibility that SLE males may have an increased predisposition or sensitivity to autonomic imbalance in the form of increased sympathetic activity (specifically rSNA), that contributes to a development of hypertension without the need for preceding renal injury. While renal nerves were not implicated in the pathogenesis of hypertension in female SLE mice [48], the possible increased sensitivity to rSNA in male SLE mice may make renal denervation a more lucrative target for ameliorating the development of hypertension in these animals.

The TULIP (Treatment of Uncontrolled Lupus via the Interferon Pathway) 2 clinical trial for anifrolumab completed phase III in August 2019, meeting its primary endpoint of "a statistically-significant and clinically-meaningful reduction in disease activity"[49]. The drug targets the type I interferon receptor, to which IFN-α binds. SLE symptoms were assessed by the British Isles Lupus Assessment Group based (BILAG-based) Composite Lupus Assessment [50], which is a clinical evaluation of lupus disease activity based on symptoms that are classified into various human body systems, and has been used since 1988 with an update in 2005 (BILAG-2004) [51]. The BILAG-based assessment does account for hypertension, but the data are combined with scores for other symptoms. There may be an additional unacknowledged benefit to anifrolumab as a potential treatment for hypertension that wouldn't respond to more traditional medications. The results of our study insinuate that the efficacy of this new therapy may differ between males and females. While both men and women were included in the TULIP trial, there is no evidence to indicate this efficacy of the drug was analyzed by sex, especially in the context of hypertension. The target of anifrolumab is the type I interferon receptor; however, induction of this pathway is largely due to a preceding activation of TLR7. The higher expression of renal TLR7 in female SLE mice indicates elements of this pathway may be hyperactive in female SLE. Accordingly, the type I interferon receptor may not be as lucrative a target for treating the symptoms of SLE in male patients with active antibodies and minimal renal involvement, creating a need to consider and develop more efficacious therapies for this population.

In conclusion, the results of this study assert sex differences in the development of autoimmune-induced hypertension. While hypertension was observed in both male and female SLE mice, only

the females displayed signs of renal inflammation and renal injury. The current project focused on increased expression of renal TLR7 in female SLE as an explanation for the increased prevalence of renal injury in these mice. While our hypothesis was confirmed, the cause of hypertension in male SLE mice is still unidentified. These data imply that some complications (specifically hypertension) in SLE result from dysfunctions in separate pathways that may not respond to a singular intervention, which may contribute to the increased prevalence of resistant hypertension in patients with SLE. While traditional medications for hypertension may help reduce blood pressure in some SLE patients, they would not effectively target the contributions of autoimmunity such as TLR7 activation and renal inflammation. Further, current therapeutics for SLE that target the TLR7-IFNα pathway may only effectively reduce complications associated with the disease in women, who make up a majority of SLE patients. However, future efforts to study and treat SLE and other autoimmune disorders will need to closely consider the effect of sex on the development of symptoms, specifically hypertension, in order to effectively treat all patients.

APPENDIX

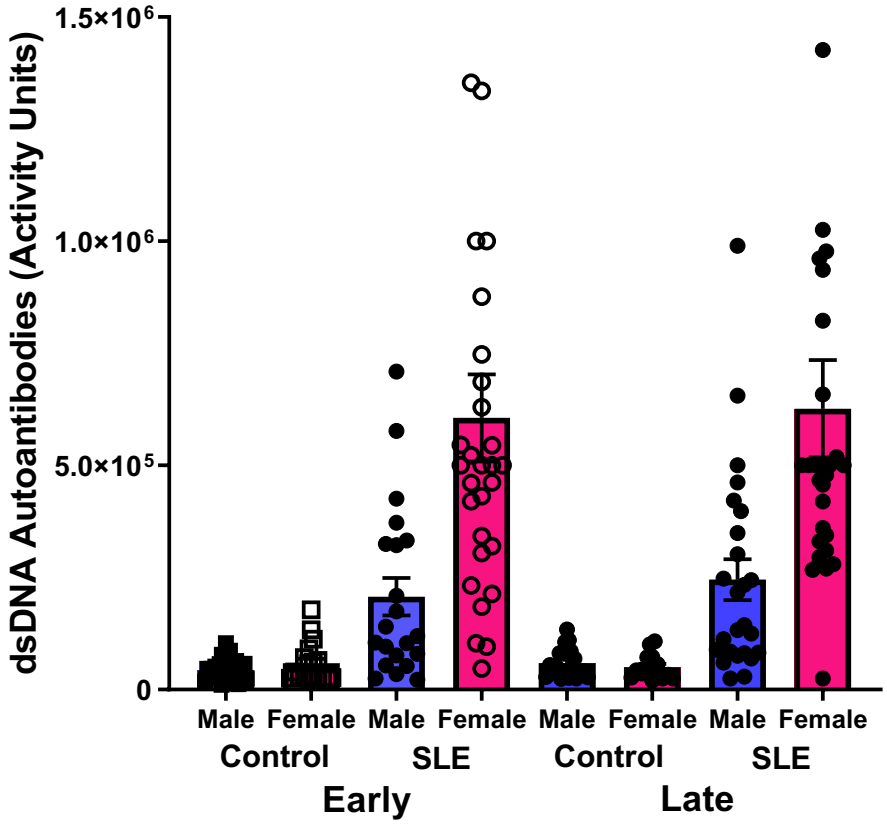

Figure 1. *Both Male and Female Mice Develop SLE*. At an early stage, female SLE mice had significantly elevated levels of double-stranded DNA (dsDNA) autoantibodies (activity units) when compared to female control mice and male SLE mice. Male SLE mice did not display higher levels of dsDNA autoantibodies when compared to either male or female control mice at the early time point. Both trends were true of the late time point as well. While there were no changes in dsDNA autoantibody levels from early to late time point, the percentage of mice that had a positive index increased from 52% to 60% in SLE male mice and 90% to 97% in SLE female mice. (n =20-29/group; group/treatment: $p < 0.001$, time: $p = 0.667$, interaction: $p = 0.999$)

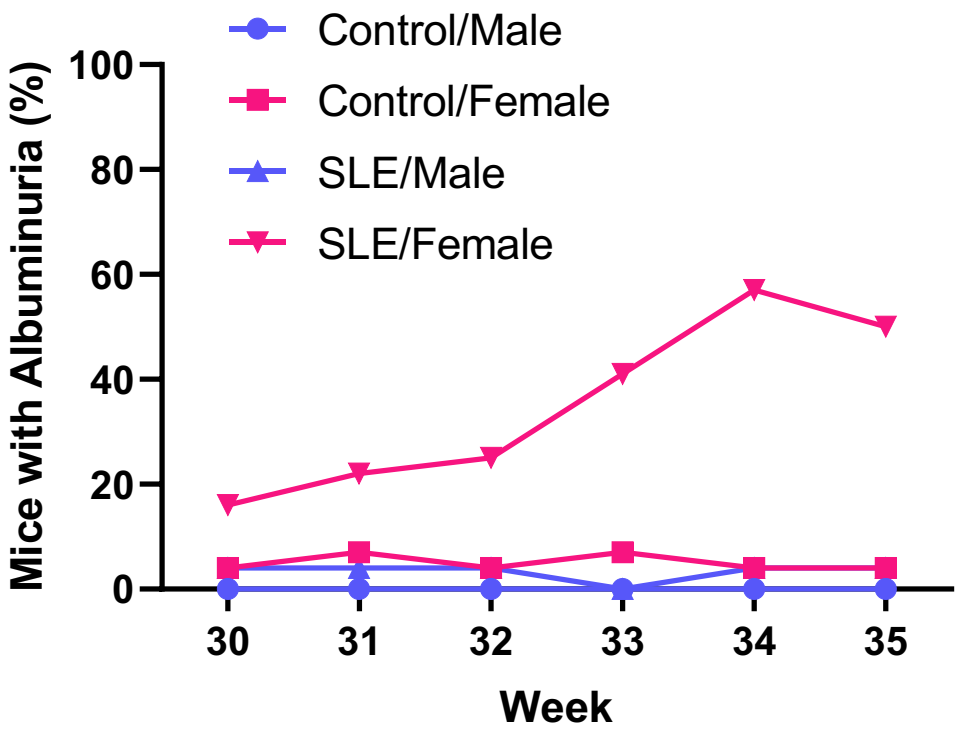

Figure 2. *Male SLE mice do not develop renal injury*. Female SLE mice developed albuminuria (urinary albumin concentration of >300 mg/dL) with a prevalence of 50% (13 out of 26) by 35 weeks. 4% (1 of 25) of SLE males and none of the male control mice developed albuminuria throughout the course of the study, while 4% (1 of 27) of female controls developed albuminuria.

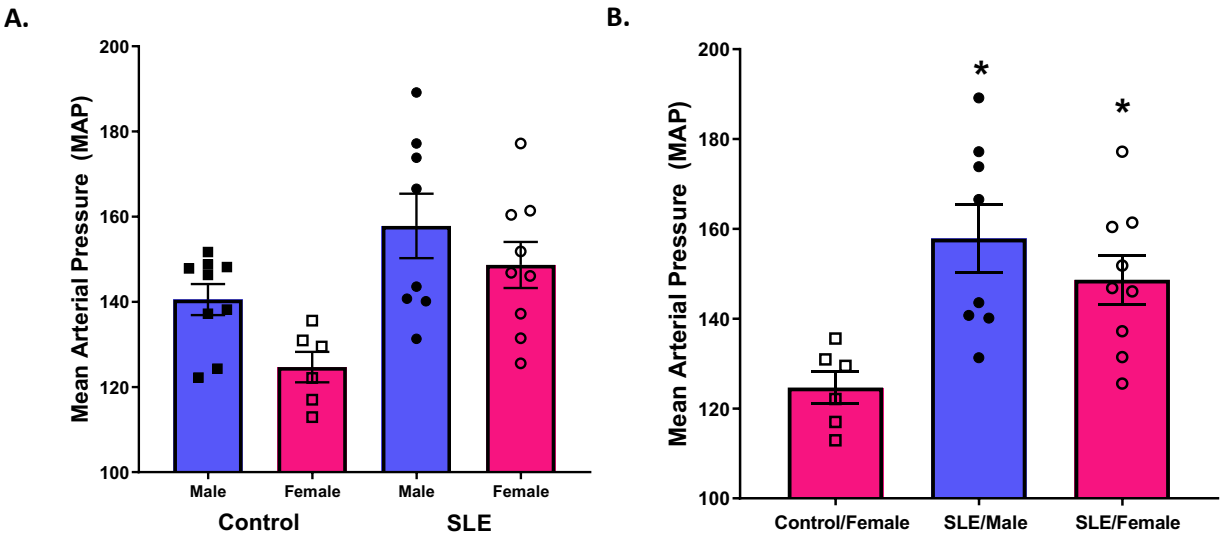

Figure 3. *Both Male and Female SLE mice develop hypertension*. At the late time point, mice were surgically implanted with a carotid catheter. After a one-day recovery period, conscious blood pressure (mmHg) was recorded for 30 minutes a day for 2 days. (A) Statistical comparisons were made using a two-way ANOVA. P- values were caluclated using the Holm-Sidak post hoc analysis. (*n* = 6-11/group; group: $p < 0.001$; sex: $p = 0.031$; interaction: $p = 0.549$). (B) The female controls have been extensively studied in previous investigations, and prove a more accurate indication of hypertension in the SLE mice. Both male and female SLE mice had significantly higher blood pressure (mmHg) than female controls. Statistical comparisons were made using a t-test. (n = 6-11/group; $p < 0.001$)

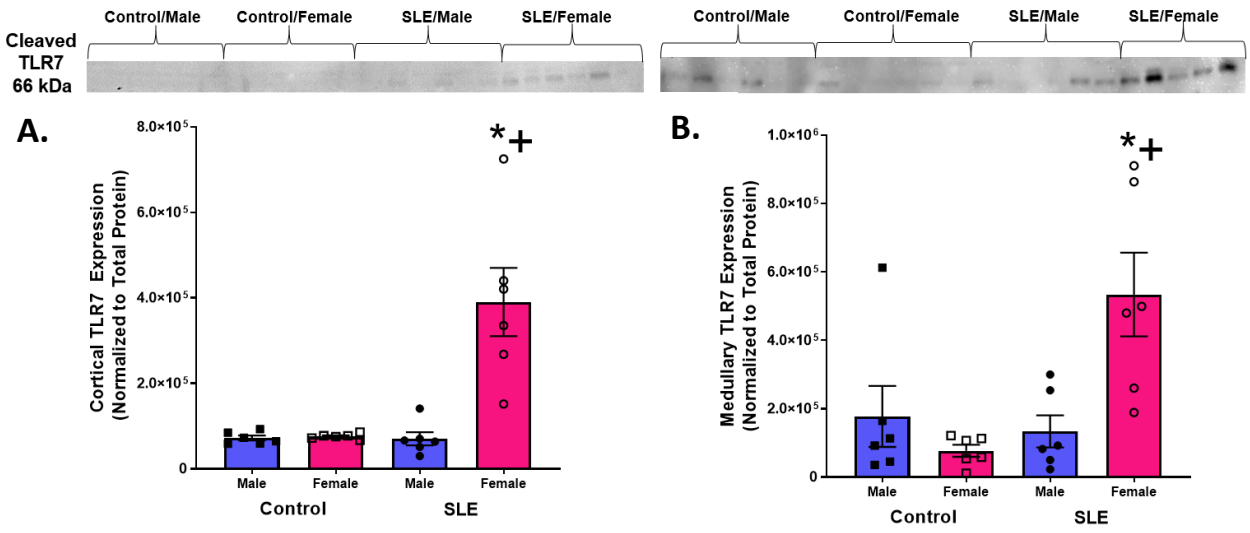

Figure 4. *Female SLE mice express higher levels of renal TLR7*. Protein expression of TLR7 was assessed via Western blot in the renal (A) cortices and (B) medullas of male and female SLE and control mice. Female SLE mice displayed a significantly higher expression (activity level) of toll-like receptor 7 (TLR7) (at 66 kDa, the cleaved version) than both male SLE and female control mice. Statistical comparisons were made using a two-way ANOVA. P- values were caluclated using the Holm-Sidak post hoc analysis. (n = 6/group; +P vs. control/female; $^{*}P$ vs. SLE/male)

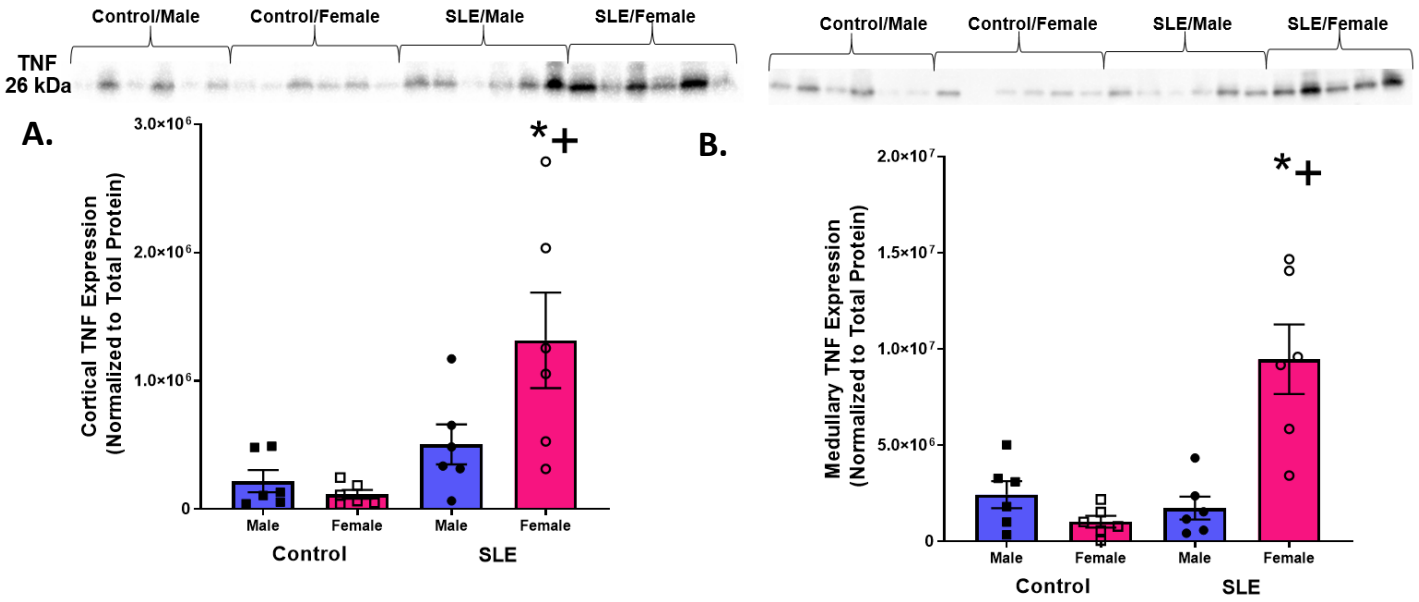

Figure 5. *Female SLE mice express higher levels of renal TNF-α*. Protein expression of was assessed via Western blot in the renal (A) cortices and (B) medullas of male and female SLE and control mice. Female SLE mice displayed a significantly higher expression (activity level) of tumor necrosis factor (TNF)-α (at 26 kDa, the transmembrane form) than male SLE and female control mice in both the renal cortex and medulla. Statistical comparisons were made using a two-way ANOVA. P- values were caluclated using the Holm-Sidak post hoc analysis. (*n* = 6/group; +*P* vs. control/female; $^{*}P$ vs. SLE/male)

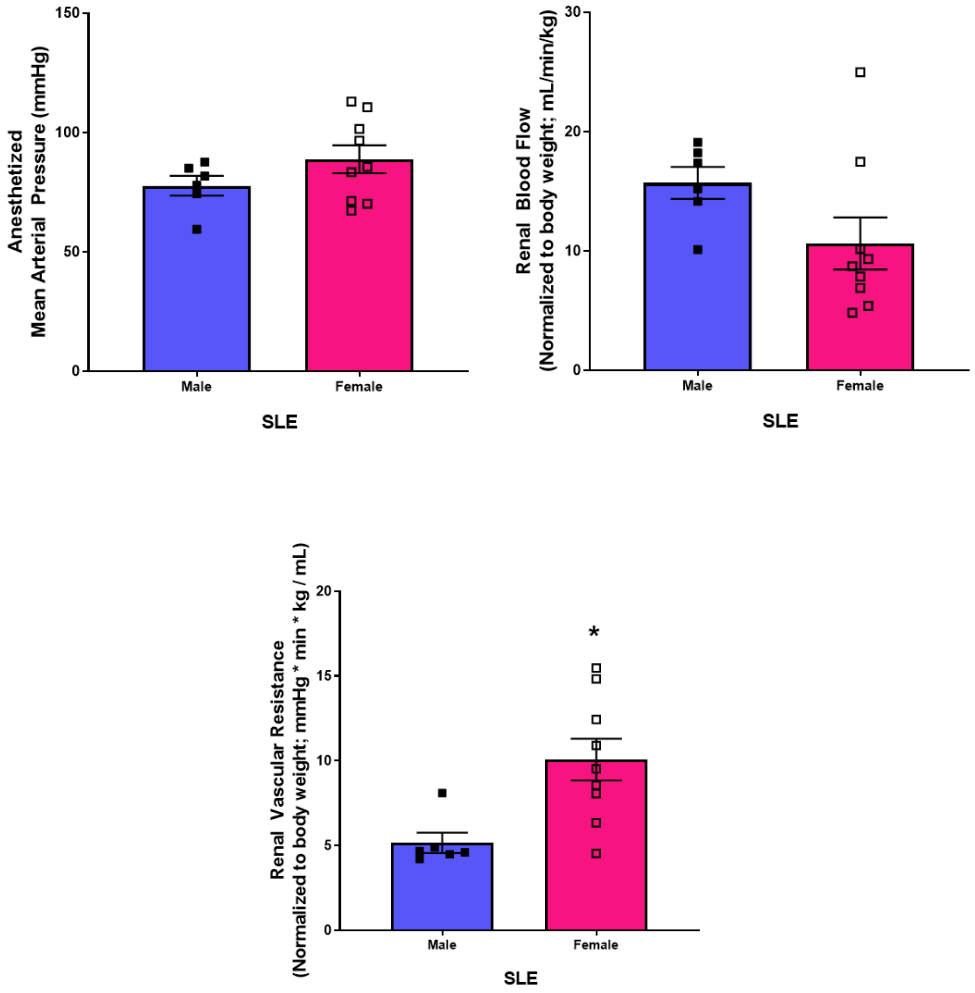

Figure 6. *Female SLE mice have higher renal vascular resistance than male SLE mice*. Conscious, anesthized mice were implanted with a catheter in the carotid artery to obtain (A) anesthetized blood pressure (mmHg). Renal blood flow (RBF, ml/min) was obtained using a Transonics probe (B). Renal vascular resistance (RVR) was calculated dividing anesthetized blood pressure by RBF. Data was normalized by dividing by body weight (g). Female SLE mice had significantly higher RVR adjusted for body weight (mmHg • min• kg • ml^{-1}) than male SLE mice (C). Statistical comparisons were made using a two-way Student's t-test. (n = 6-9/group; $^{*}P$ vs. SLE/male)

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