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Angiotensin II: A Potential Link Between Inflammation and Hypertension in SLE

Khush Singh Aujla

Angiotensin II as a Potential Regulator of Inflammatory Cytokines in Inducing Hypertension in Systemic Lupus Erythematosus

Khush Singh Aujla

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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Approved by

Advisor: Professor Mika Jekabsons

Reader: Professor Christopher Leary

Reader: Professor Donna West Strum

Dedication

I would like to dedicate my thesis to my sister, Navketan Kaur Aujla. Your guidance has helped me reach so many of my dreams, and your motivation pushes me to go even higher. No song better describes our relationship.

Hindi:

Phoolon Ka Taaron Ka Sabka Kehna Hai

EK Hazaron Mein Meri Behna Hai

Sari Umar Hame Sang Rehna Hai

Translation:

Flowers and Star, All Of Them Say

One In A Thousand Is My Sister

All Our Lives We Will Be There For Each Other

Acknowledgments

I would like to acknowledge Dr. Michael Ryan and Dr. Keisa Mathis for mentoring me through my research at the Physiology Department at the University of Mississippi Medical Center, Dr. Mika Jekabsons for advising me through my thesis writing, and the Sally McDonnell Barksdale Honors College for giving me the opportunity to challenge myself.

Abstract

Khush Singh Aujla: Angiotensin II as a Potential Regulator of Inflammatory Cytokine Induced Hypertension in Systemic Lupus Erythematosus.

(Under the direction of Dr. Mika Jekabsons)

The disease Systemic Lupus Erythematosus is a chronic inflammatory, autoimmune disorder that primarily affects women during their reproductive years. Women with SLE are at a greater risk of developing hypertension, which increases their risk of mortality from a cardiac related event. A proposed mechanism for SLE hypertension suggests that inflammation in the kidneys causes renal dysfunction, presumably resulting in less water excretion, increased plasma volume, and thus high blood pressure. This experiment tests the hypothesis that the T-cells from a mouse model of SLE hyper secrete the inflammatory cytokines IL-17, IL-10, and IFN- γ, and are more sensitive to the cytokine-stimulating hormone angiotensin II (ANG II). To test this hypothesis, T lymphocytes were isolated from control and SLE mice, and cytokine secretion into the culture media was determined in the presence or absence of ANG II. Thymocytes from SLE mice secreted greater levels of all three inflammatory cytokines, although excess IL-17 secretion occurred only after the onset of renal damage. Angiotensin II increased production of IFN- γ, but there was no major difference between the SLE and Control groups. These results indicate that hyper secretion of IL-17, IL-10, and IFN- γ by SLE T-cells may be contributing to renal inflammation, kidney damage, and therefore SLE hypertension. T-cell hypersensitivity to ANG II could not account for the hypertension, suggesting that these cells are excessively sensitive to another factor (that is present in the culture media) or have an innately higher secretion rate.

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List of Abbreviations

SLE	Systemic Lupus Erythematosus
RVR	Renal Vascular Resistance
GFR	Glomerular Filtration Rate
RBF	Renal Blood Flow
ANG II	Angiotensin Two
MMF	Mycophenolate Mofetil
T-cells	Thymus Maturing Lymphocytes
B-cells	Bone Marrow Maturing Lymphocytes
IL-10	Interleukin Ten
IL-17	Interleukin Seventeen
IFN- γ	Interferon Gamma
Na^+	Sodium Ion
NZBWF1	SLE Mouse Model
NZW	Control Mouse Model
MAP	Mean Arterial Pressure
RPMI	Cell Nutrient Buffer
ACK	Lysis Buffer
CD4+	T-cell Surface Binding Site
uL	Micro Liter
ELISPOT	Enzyme Linked Immunospot Assay
g/ml	Grams Per Milliliters

Introduction

Systemic Lupus Erythematous (SLE) is a chronic inflammatory, autoimmune disorder. In this disease immune cells mistakenly identify their own host tissue as foreign threats, and cause excessive inflammation. Prolonged inflammation leads to swelling and continued recruitment of immune cells, which degrade the body's cells and tissues (1). A positive diagnosis for SLE is confirmed if at least four of the criteria listed by the American College of Rheumatology are met (Table 1). The symptom most often exhibited by SLE patients is a malar rash, which creates a butterfly pattern across the cheeks and nose (1). This distinct feature is also similar to a wolf's facial design, giving the disease its trademark name, Lupus.

SLE is a complex disease that can potentially affect any organ system during a *flare,* or a period of elevated disease activity. Alternatively, in some patients it can be effectively managed such that long periods of remission are possible (2). SLE is a gender specific disorder, with females about nine times more likely to develop the disease. Furthermore, the disease is most commonly active during women's reproductive years. The pathogenesis of SLE remains unclear, but it is likely that several genetic, environmental, and hormonal factors may be involved (3). Current treatments for SLE target decreasing the frequency of *flares*, and reducing their inflammatory effects once they occur (2).

For many patients' advances in general medical care has improved the short-term prognosis for SLE. Yet there is still no cure for this disease, and the chronic inflammation

Criterion	Description
Malar rash	"Butterfly" shaped rash across the cheeks
Discoid rash	Skin rash in the shape of a coin or an oval
Photosensitivity	Skin rash caused by a reaction to sunlight
Oral ulcers	Oral or nasopharyngeal ulcers
Nonerosive arthritis	Tenderness, swelling, or effusion of peripheral joints
Pleuritis, pericarditis	Inflammation or effusion of the covering protecting the heart and lungs
Renal disorder	Proteinuria, cellular casts
Neurological disorder	Seizures, psychosis
Hematologic disorder	Hemolytic anemia or leukopenia
Immunologic disorder	Anti-DNA, anti-Sm, or anti-phosholipid antibodies
Positive antinuclear antibody	Anti-nuclear antibodies

Table 1: Diagnostic Criteria for SLE. List of common criteria used in diagnosing Systemic Lupus Erythematosus, based on recommendations from the American College of Rheumatology. A positive diagnosis of lupus is commonly confirmed if four of the listed criteria are present. Most patients initially present with the malar rash on the face, or the discoid rash throughout the skin. The most prominent sign of SLE is the presence of autoimmune antibodies in the blood; the immunologic disorder is seen in almost all SLE patients. If untreated the disease can lead to inflammation in several different areas (brain, kidney, joints...etc) and cause severe complications (psychosis, nephritis, arthritis, etc...) in those areas. (Smith)

can degrade tissue structure throughout the body, resulting in loss of organ function (2). Trager and Ward found that the probability of survival for SLE is 95% at 5 years, but 78% after 20 years (4). The primary cause of mortality after the first five years is cardiovascular disease, with dysfunctions found in the heart and/or blood vessels. Women between 15 and 44 years of age with SLE are at a 50% greater risk of dying from a cardiac related mortality than their age-matched healthy counterparts (5). One of the major risk factors for a cardiac event is hypertension, a medical condition characterized by chronically high blood pressure clinically diagnosed as systolic/diastolic pressures greater than 140/90 mmHg. Elevated blood pressure causes the heart to work harder as a greater force is required to pump blood against the higher pressure. The greater workload increases the risk of heart failure. Additionally, high arterial pressure increases the risk of stroke stemming from rupture of an artery (6). Studies have shown that women with SLE have a much greater chance of developing hypertension than women without SLE (7). Despite a stark contrast, little is known about the mechanisms behind the high prevalence of hypertension in SLE patients.

The kidneys play an important role in the long-term regulation of blood pressure by regulating plasma volume. Therefore, it is important to consider their function during any condition that leads to hypertension. Through a controlled balance of excretion and absorption, the kidneys regulate blood fluid volume, electrolyte levels, osmolarity, and acidity. Each kidney appears as a bean-like structure, and is connected to the bladder by the ureter. The renal circulation receives a fifth of cardiac output through the renal artery (8). Within the kidney are functional units known as nephrons. Blood enters the nephrons by the afferent arterioles, and is partially filtered into nephron tubules by the glomerular

capillaries, while the unfiltered blood exits through the efferent arterioles. The renal vascular resistance (RVR) to blood flow in the afferent arteriole controls the blood flow into the glomerular capillaries. The nephron tubules into which the filtered blood enters, consist of the following segments in the order the filtrate circulates through them: the Bowman's capsule, the proximal tubule, the loop of Henle, the distal tubule, and the collecting duct (Figure 1). The filtrate is formed when bulk flow moves fluid out of the plasma along with small electrolytes and waste. Plasma proteins like albumin and red blood cells are too large to pass through the Bowman's capsule, and their presence in the urine usually indicates kidney dysfunction (9). The glomerular filtration rate (GFR) is a key measure of kidney function, and is determined by analyzing the amount of plasma filtered per minute. RVR partly determines GFR by affecting glomerular capillary pressure, a major force driving filtration. The balance of filtered water in the nephron that is excreted or reabsorbed in to the plasma ultimately determines blood volume and thus blood pressure (8).

Since the kidneys are the long-term regulators of blood pressure, hypertension is accompanied by a change in renal hemodynamics. One of the main symptoms of SLE is lupus nephritis, in which the immune system attacks the kidney tissue. The condition is categorized in six morphological classes depending on severity, and it is estimated that as high as 50% of patients with SLE can develop some form of lupus nephritis (10). As immune cells attack the kidney, one consequence can be destruction of glomerular capillaries and Bowman's capsules, which will effectively decrease kidney GFR if a critical number are lost. Hence less water will be filtered for excretion, potentially

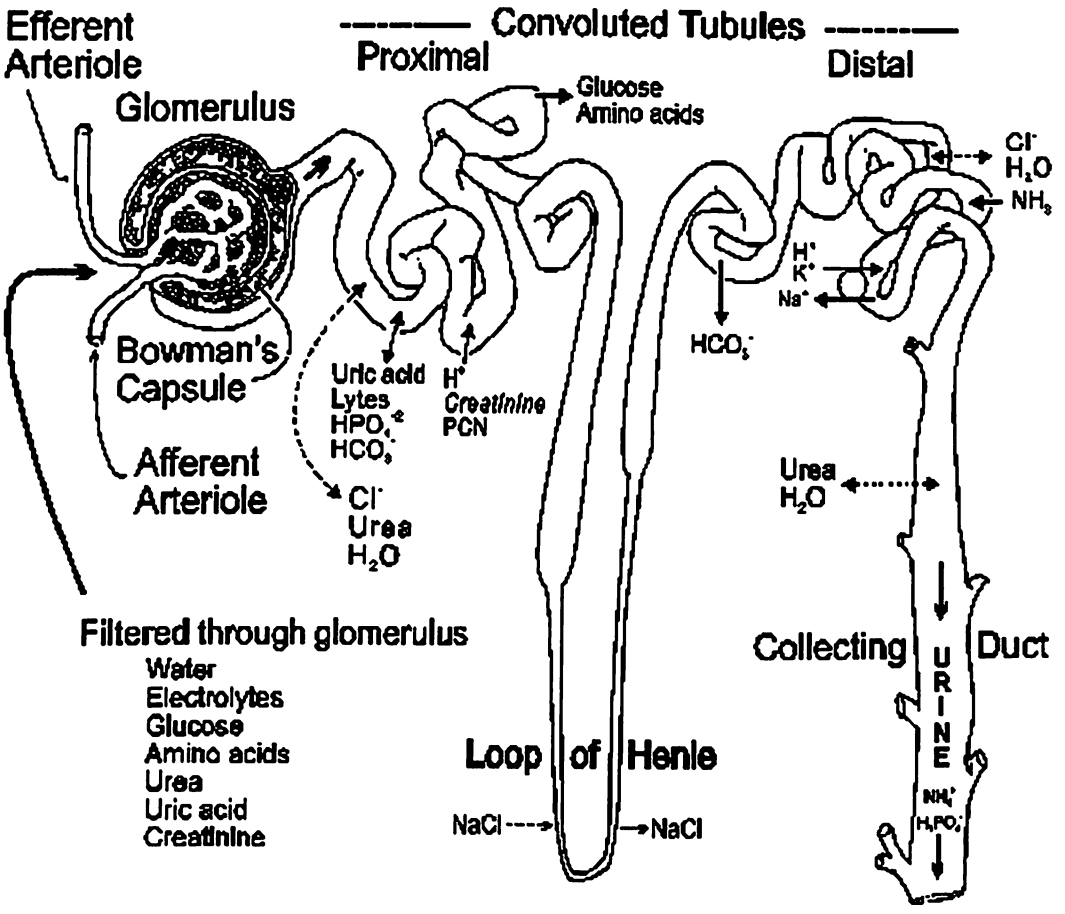

Figure 1: Diagram of the Nephron. Blood arrives at the glomerulus for filtration through the afferent arteriole. About 25% of the plasma passes through the filtration barrier to become the glomerular filtrate in Bowman's capsule. Blood cells, most of the proteins, and about 75% of the fluid leave the glomerulus via the efferent arteriole. Filtration and reabsorption of different molecules occurs throughout the convoluted tubules. The remaining waste is gathered in the collecting duct and is sent to the ureter (illustration reproduced from reference 8). During SLE inflammation' T-cells are seen surrounding the glomerulus, afferent arterioles, and convoluted tubules.

causing increased plasma volume, and thus increased blood pressure (Figure 2). Nakano et al showed that reduction in GFR correlated with the severity of lupus nephritis (11). Since this condition affects blood pressure, it is easy to assume that SLE hypertension is only dependent on nephritis. However, not all patients with SLE develop nephritis, but can still be at risk of developing hypertension. It has been shown that hypertension correlated strongly with SLE patients that had dysfunctions in their renal hemodynamics, but did not have lupus nephritis (12). This suggests that there are additional physiological mechanisms contributing to hypertension in SLE, which change renal hemodynamics in another way then nephritis. Yet, previous research has demonstrated that this change is occurring through a reduction in GFR and RBF (11).

The underlying cell and molecular mechanism responsible for the renal dysfunction in patients with SLE hypertension remains unclear. Given the autoimmune nature of this disease, it is has been hypothesized that the immune system plays a role (13). Several experiments have shown a potential role for the immune system in models of hypertension. It has been shown that inflammation may be a major contributing factor to the development of Angiotensin II (ANG II) model of hypertension as well as kidney damage in rats. Carretero et al showed that by knocking out a gene for the CCR2 chemokine receptor, which is important in the recruitment of macrophage cells to the kidneys, ANG II infused hypertension and kidney damage were attenuated (14). Experiments have shown that immunosuppressant agents such as mycophenolate mofetil (MMF) are able to suppress many of the symptoms of hypertension in rat models (15). Mattson et al showed that MMF reduced levels of T lymphocytes (T-cells) as well as B lymphocytes (B-cells) infiltrating the kidney, and also significantly decreased the blood

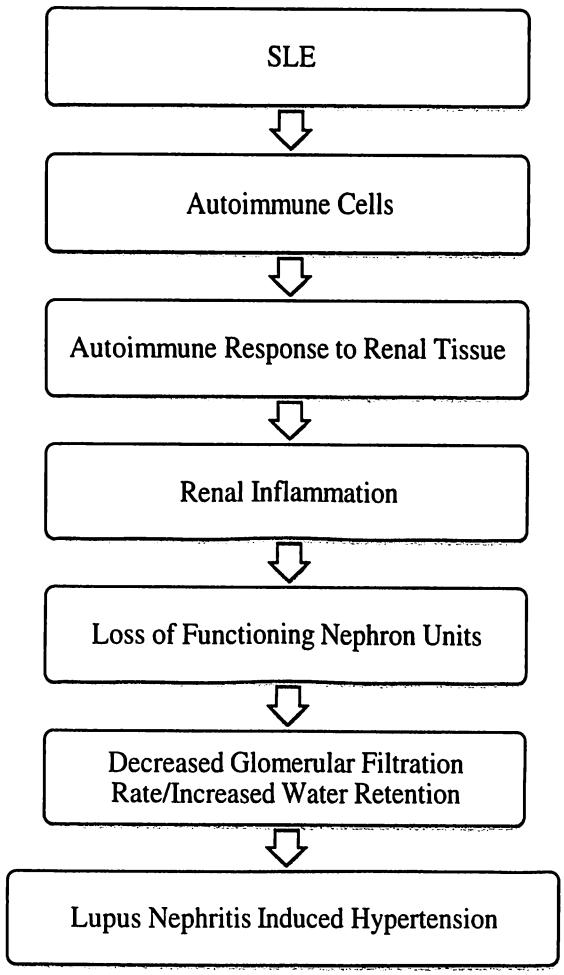

Figure 2: Mechanism for lupus nephritis induced hypertension. SLE patients have autoimmune cells, or cells that are more prone to have an inflammatory response to host tissue. The immune cells create antibodies that target renal tissue, which leads to inflammation in the kidneys. The inflammation can damage several parts of a nephron, including the Bowman's capsule and glomerular capillaries. The degradation of the nephron will lead to a decrease in glomerular filtration rate, and therefore more water retention by the kidneys. The increase in plasma volume will raise blood pressure above 140/90 mmHg systolic over diastolic, and therefore induce hypertension.

pressure in comparison to rats treated with vehicle. The immune suppression was also shown to attenuate kidney damage, indicated by decreased urine albumin levels as well as less glomerular damage (16). T-cells are suspected to play an important role in connecting inflammation and hypertension. Experiments on the Dahl salt sensitive rat model of hypertension showed that T-cells played an active role in the progression of hypertension as well as kidney damage. In this model, increased renal infiltration of T-cells correlated with increased blood pressure, increased albumin in the urine, and resulted in renal glomerular and tubular damage (17). Since autoimmune responses are the basis of inflammation in SLE, immunosuppression to attenuate the inflammation is likely to reduce the degree of hypertension. However, suppressing inflammation once it has started still leaves the patient susceptible to damage. Instead it is potentially more beneficial to target the mechanism that triggers inflammation in SLE.

Immune cells are initially activated by antigens, and secrete several different cytokines into the circulation to recruit more cells thus producing inflammation. Cytokines are cell-to-cell paracrine factors that work by binding to specific cell surface receptors, and altering the cell's function. This can include changing the expression of a gene by affecting transcriptional factors, or causing the production or suppression of other cytokines. The effects on the cell vary according to cell type and by individual cytokines, but all cytokines fall into distinct family classifications (18). It has been shown that several inflammatory cytokines are increased in the tissues and plasma of patients with SLE, suggesting that they may play a role in SLE hypertension (13). In this respect, three cytokines of interest are interleukin 17 (IL-17), interleukin 10 (IL-10), and interferon gamma (IFN- γ). IL-17 is known to be a pro-inflammatory cytokine that

stimulates certain immune cells to produce other cytokines, and is linked to several autoimmune diseases (19). Experiments have shown that IL-17 is increased in the plasma of SLE patients. This has lead to the hypothesis that IL-17 plays an important role in the pathogenesis of SLE (20). The cytokine IL-10 is actually considered an anti-inflammatory cytokine because it down regulates the activity of T-helper cells. However IL-10 activates B-cells to produce antibodies, which in an autoimmune disease, become auto antibodies that mistake host tissue as threats. This in turn causes a greater inflammatory response than the original anti-inflammatory benefits of IL-10 (21). It has been shown that the plasma level of this cytokine by T-cells is increased in patients with SLE, and the plasma levels have positively correlated with the severity of the SLE (22). The pro-inflammatory cytokine IFN- γ stimulates other immune cells during inflammation, and experiments have shown an increase in the cytokine levels in certain blood cells from patients with SLE (23). New approaches for SLE treatment include therapies where IFN- γ related genes are suppressed (24).

There are at least two possible mechanisms that can account for the increased plasma levels of these cytokines. First, a greater number of immune cells may be present, so that the collective rate of cytokine secretion into the plasma is greater. Second, the existing population of immune cells may be hyperactive, and thus secreting more cytokines per cell. My hypothesis is that the later explanation is true, although these hypotheses may not be mutually exclusive. This surplus of cytokines may recruit additional immune cells into the kidneys that increase renal inflammation, which impairs the renal regulation of blood pressure, and ultimately leads to hypertension. The goal of this study was to determine the mechanisms responsible for this overproduction.

The hormone ANG II may be a potential cause for the increase in inflammatory cytokines in SLE hypertension. ANG-II is a part of the renin angiotensin system, and is a major regulator of blood pressure. The molecule works by indirectly increasing the reabsorption of Na^+ by the distal tubule as well as the collecting duct, which leads to water reabsorption through osmosis. It can also reduce GFR and the plasma flow through the kidneys by causing arteriole vasoconstriction. ANG-II binds to specific receptors on cell surfaces, which are common drug targets to lower blood pressure (8). Chronic infusion of ANG-II has been shown to induce hypertension in mice. Furthermore, experiments have supported the idea that ANG-II at least partially promotes hypertension by affecting T-cells (25). One experiment showed that mice, which were genetically engineered to lack T and B-cells, did not become hypertensive under chronic ANG-II infusion. Yet, when only the T-cells were administered back into the knockout mice, and ANG-II was chronically infused, the hypertension returned. (26). It is thought that by activating the T-cells, ANG-II is inducing the production of inflammatory cytokines. It has been shown that ANG-II increases the production of pro-inflammatory cytokines when inducing hypertension (27). Similarly, the blocking of the ANG-II receptor has been shown to have anti-inflammatory effects by decreasing the production of the pro-inflammatory cytokine IFN- γ (28).

In this study, an additional hypothesis to be tested is that the hormone ANG-II increases the production of the inflammatory cytokine IFN- γ by T-cells. There is no conclusive evidence that ANG-II levels are elevated in patients with SLE. Yet ANG-II is seen to both correlate with the production of certain inflammatory cytokines by T-cells, and with the presence of hypertension. Since SLE is an inflammatory disorder with an

increased risk of hypertension, my last hypothesis is that ANG-II will increase the production of inflammatory cytokines to a greater extent in SLE T-cells. A proposed model for the role of T-cells, ANG II, and inflammatory cytokines in the development of SLE hypertension is outlined in Figure 3.

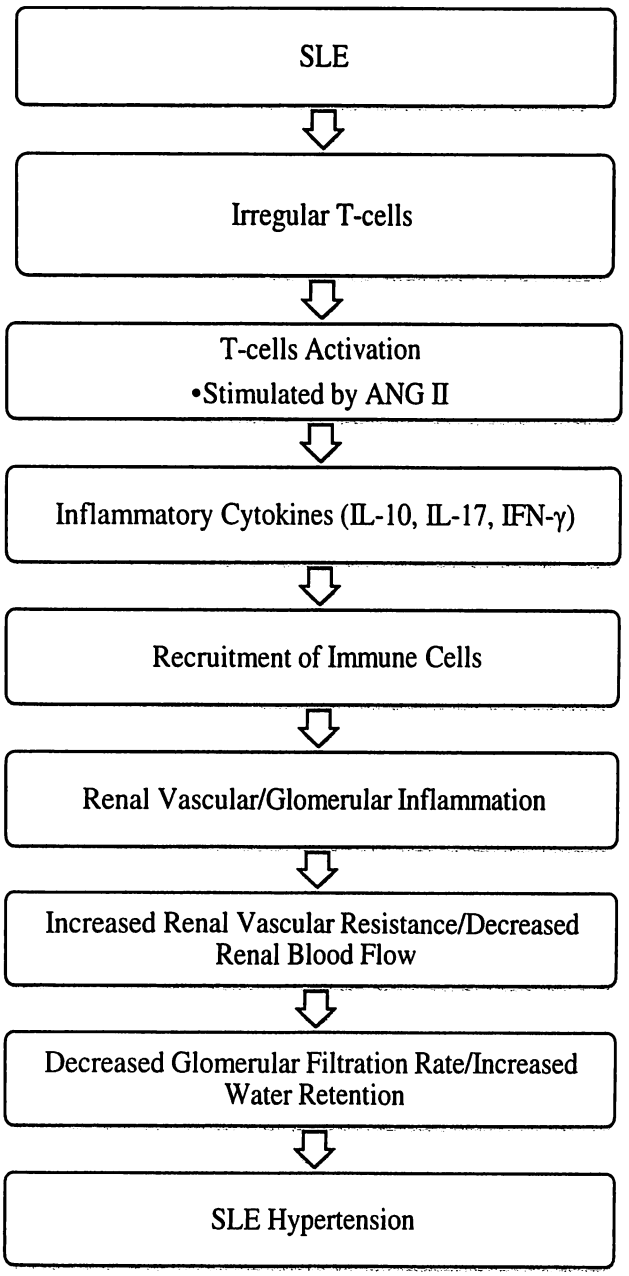

Figure 3: Proposed Mechanism for SLE Hypertension. SLE patients have irregular T-cells, or cells that are more prone to produce an inflammatory response. A possible trigger for SLE T-cell activation in the kidneys is ANG II. The T-cell activation may lead to an increase in the production of inflammatory cytokines, which recruit other immune cells to the kidneys through circulation. This leads to inflammation in the renal vasculature, which elevates RVR, and therefore a decrease in renal blood flow. Without adequate blood flow to the glomerulus, the GFR decreases, and thus the kidneys excrete less water. The increased plasma volume results in a rise in resting blood pressure above 140/90 mmHg systolic over diastolic. If the elevated blood pressure becomes chronic, hypertension develops.

Specific Aims

This study tests the hypothesis that T-cells from a mouse model of SLE secrete more of the inflammatory cytokines IL-17, Il-10, and IFN- γ than T-cells from non-SLE control mice, and that ANG-II increases the production of IFN- γ to a greater extent in SLE then Control T-cells. The specific aims were to:

(i) Isolate T-cells from control and SLE mice.

(ii) Determine the baseline secretion of IL-17, IL-10, and IFN- γ in a defined cell culture medium.

(iii) Determine the sensitivity of T-cells' IFN- γ secretion to ANG-II.

(iv) Compare IFN- γ secretion changes in the presence of ANG-II between SLE and Control T-cells.

Materials and Methods

Animals

Dr. Michael Ryan (Department of Physiology, University of Mississippi Medical Center) provided female strains of mice for the experiment. The offspring of a cross between the New Zealand Black crossed and the New Zealand White (NZBWF1) mice al spontaneously developed SLE, the origin of which is unknown. NZBWF1 mice exhibit several characteristics similar to human SLE, most importantly their development of hypertension (Figure 4). The NZW (Control mouse) model was used to represent healthy conditions. This model is a common parental control for the NZBWF1 model, and does not display characteristics of human SLE. Both strains had their urine albumin levels measured regularly as an index of renal function, with trace levels meaning the lowest detection limit and 2000 mg/L the highest. After thirty-six weeks' the NZBWF1 mice began to develop hypertension, with blood pressure elevating close to 140/90 mmHg systolic over diastolic, at which point both groups were sacrificed.

Mouse spleenocyte (spleen immune cells) isolation.

(All buffers and solutions used to isolate the Spleenocytes were from the Mouse Spleen T-cell Isolation Kit by Life Technologies.) Each mouse was prepared for dissection, and cleaned with 70% ethanol. The abdominal cavity was cut open, and the spleen was removed and placed in a cell strainer. The spleen was then mashed through the strainer into a petri dish, and the remaining cells in the strainer were rinsed out with 5 ml of RPMI (cell culture) buffer. The cell suspension was transferred into a 15 mL

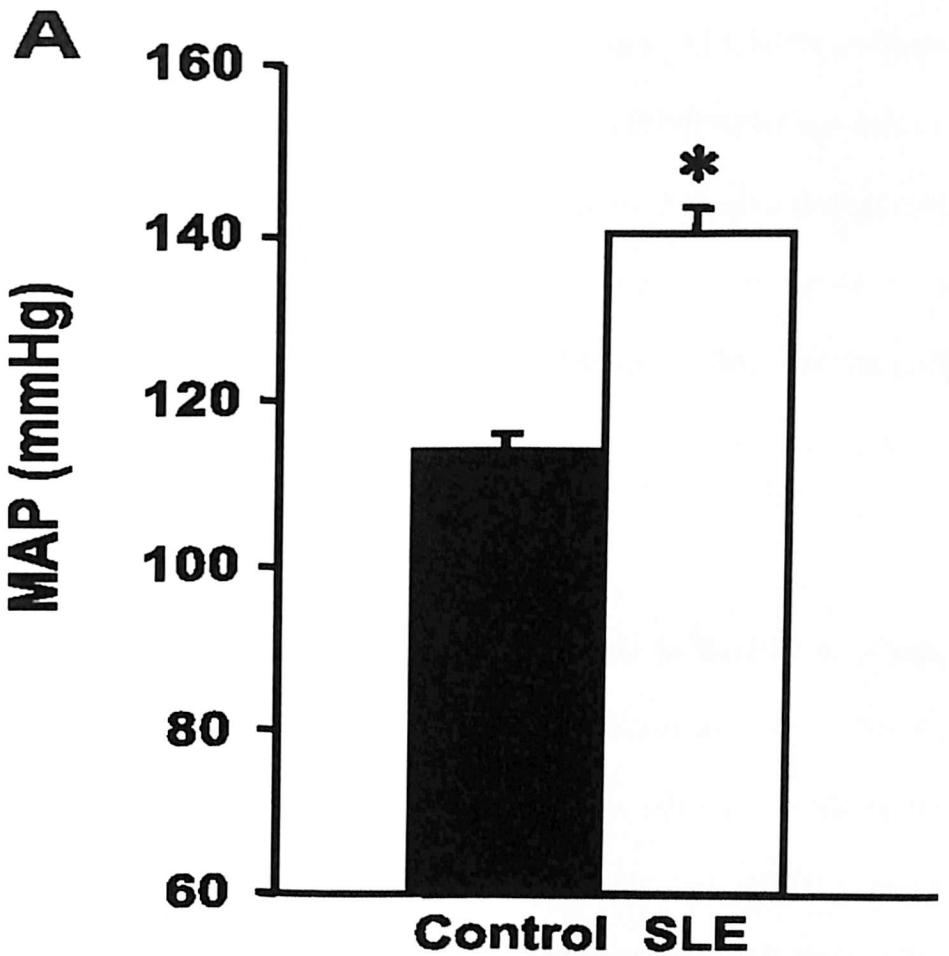

Figure 4: Mean Arterial Pressure for Control and SLE Mice. The data in this figure is taken from reference number 13, and represent published data from Dr. Michael Ryan (University of Mississippi, Department of Physiology). Comparing the mean arterial pressure (MAP) in mmHg between female NZBWF1 and NZW mice. On average the SLE mice had significantly higher blood pressure (140 mmHg) then the control mice (115 mmHg).

conical tube, and centrifuged at 1600 revolutions per minute (rpm) for 9 minutes. The cell pellet of spleen cells was suspended in 1 ml of an ACK buffer, to lyse all the red blood cells in the pellet. Afterwards another 9 ml of RPMI buffer was added, and the mixture was centrifuged. The supernatant was discarded to remove all dead cells. The remaining pellet consisted only of viable spleenocytes, and was resuspended in 3 ml of RPMI buffer. The sample was run through a cell counter to determine the amount of cells that had been isolated.

Dynabeads Mouse CD4+ T-cell Isolation.

(All buffers and solutions used to isolate the T-cells from spleenocytes were from the Dynabeads Mouse CD4+ T-cell Isolation Kit by Invitrogen.) Dynabeads are protein A-coated microscopic beads that bind to mouse antibodies T-cell specific CD4+ plasma membrane receptors. After the spleenocytes were counted, they were washed again and then resuspended at one million cells in 500ul of isolation buffer and 25ul flowcomp mouse CD4 antibody. The mixture was stirred and incubated at 2 °C for ten minutes. Afterwards 2ml of isolation buffer were added to wash the cells, followed by centrifugation, and removal of the supernatant. The cell pellet was resuspended in 1 ml of isolation buffer, and transferred to a 1.5 ml eppendorf tube. Protein A Dynabeads, 75 µL were washed and then transferred to the cell suspension. After fifteen minutes of incubation, the tube was placed on a magnet. The Dynabeads with the CD4+ T cells attached were retained in the tube by the magnet, while the remaining supernatant containing other splenocytes was discarded. The Dynabeads were then washed twice in isolation buffer. Next the Dynabeads were resuspended in 1 ml of flowcomp release buffer, which caused the cells to detach from the antibodies. The cells were then remixed

and replaced on the magnet. This time the cells remained in the supernatant and only the Dynabeads with the attached antibodies adhered to the tube by the magnet. The supernatant containing the cells was removed and placed in a 15 ml tube. Next 2 ml of isolation buffer was added and the suspension centrifuged. The supernatant was discarded and the remaining pellet was resuspended in 2 ml of RPMI buffer. The remaining solution contained only CD4+ T-cells. A sample of the solution was run through a cell counter to determine the number of CD4+ T-cells that had been isolated.

CD4+ T-cell activation and culturing.

To activate the T-cells to produce inflammatory cytokines, they had to be stimulated. Each cell culture was prepared by mixing 1 million T-cells with a cell culture media, consisting of 10% fetal bovine serum in RPMI buffer supplemented with 10 μM IL-2 to stimulate T-cell cytokine production. To determine the amount of media to use per sample, calculations were based on the population of T-cells isolated (calculations A). The combined T-cells media was then plated into each well of a 8 well chamber. To stimulate mitosis, 25μL of CD3/CD28 Dynabeads were added to each well of cell culture. The cells in the culture were allowed to attach to the bottom surface, over 48 hours in 5% CO_2, 37°C incubator. After incubations, the cells had proliferated to greater quantities, and released a mixture of inflammatory cytokines into the media (figure 5).

Measurement of inflammatory cytokine production.

After incubation different assays were run on the culture media to measure cytokine levels. IL-17 and IL-10 were measured using Mouse IL-17 and IL-10 immunoassays from Quantikine. The assays were prepared in a 96 well plate, and all

reagents were included in the assay kit. To each well 50μL of either a standard sample, a control, or an unknown sample was added with 50μL of assay diluent. The wells were incubated for 2 hours, washed, and then 100μL of conjugate was added to each well. After another wash, 100μL of substrate solution was added to each well, and incubated for 30 minutes. The substrate solution caused the samples to change color based on the amount of the cytokine they contained. Adding a stop solution stopped the reaction, and then the 96 well plate was read in a spectrophotometer at 570 nm.

The IFN- γ was measured using an ElISPOT assay. The assay was run in a 96 well Millipore plate, and all reagents used were included in the assay kit. First the plate was coated with 100mL of coating antibodies at 15mg/ml, and incubated overnight. Next a 100μL of the standards, controls, and T-cell media samples were added to each well, and incubated for 40 hours. After incubation the wells were washed six times in distilled water. A 100μL of 1mg/ml diluted IFN- γ was added to each well, and incubated for an hour. A 100μL of blocking buffer was added to each well, and incubated until dark spots appeared on the plate bottoms. The plate was then washed in distilled water, and the spots were counted using an automated machine. Due to time and resource constraints only the IFN- γ protocol was repeated in the presence of ANG-II. Half of the cell plates from both the SLE and control group received 10 uM of ANG-II (calculations B). An IFN- γ ELISPOT assay was repeated to measure the effects of ANG-II. All figures were calculated using Microsoft Excel.

Calculations

A. T cells and culture media

- Count isolated T cells (cells/ml)
- Plate each well (5 wells per sample) with 1 million T-cells in a total volume of 1.5 ml

((# Isolated T-cells)/ (1e^6 cells)) = ((1.5 ml)/ (X)) X = volume of cells per well

(1.5 ml) – (volume of cells per well) = (volume of media per well)

((Volume of cells per well) / (1.5 ml)) = ((volume of media per well) / (X))

X = total volume of culture media needed.

Example:

6.5e6 cells / 1e6 cells = 1.5 ml / X X = 230 ul (volume of cells per well)

1.5 ml – 230 ul = 1, 270 ul (media per well)

230 ul / 1, 500 ul = 1,270 ul / X X = 8, 282 ul (media total)

- Combine total volume of media needed directly to the volume of T cells

Plate each well with 1.5 ml of the combined T cells and media

B. Angiotensin II (Molecular Weight = 1046.18 g/mol)

- Add 10.4 mg of ANG II into 10 ml of distilled water for a stock solution (concentration = 10mM). Dilute the stock solution, take 1 ml and add to 9 ml of distilled water for a work solution (concentration = 1mM).

Add 20ul of work solution into half of the wells (final concentration added = 10 uM)

Results

IL-10 secretion is greater from SLE then Control T-cells.

Figure 5 illustrates the difference in IL-10 secretion by SLE and Control T-cells stimulated by IL-2 and Dynabeads. The two SLE mice had severe albumin (2000) levels, while the two control mice had minor (trace). Analysis of the IL-10 immunoassay showed that IL-10 secretion for SLE T-cells was almost four times that of Control T-cells, 243 pg/ml to 65 pg/ml. This suggests that SLE T-cells are producing IL-10 at a greater rate then healthy T-cells in the presence of IL-2 and Dynabeads.

IL-17 secretion is similar between Control and SLE T-cells with low albumin, but higher in SLE T-cells with high albumin.

An immunoassay for IL-17 originally showed minor differences between the Control and SLE group, but there was a major difference between the two T-cell preparations from the SLE mice. One mouse had similar IL-17 levels to control, while the other had double the concentration production. When checking previous notes it was apparent that the SLE mouse with higher IL-17 levels was the mouse with very high levels of albumin in the urine. The urine of the mice had been checked daily, and albumin levels were indicated on a rudimentary scale that measured from trace (none) to 2000 (severe). Figure 6 illustrates that greater level of albumin in the urine correlated with greater IL-17 production. The Control mice had no measurable Albumin, while one SLE mouse had an albumin level of 100 (minor). The two groups had approximately the same levels of IL-17 production by their T-cells, 13.4 pg/ml to 13.7 pg/ml. As for the mouse with severe albumin in the urine, it's IL-17 production of 27.3 pg/ml was double that of the Control group and the other SLE mouse. This suggested that SLE T-cells do not

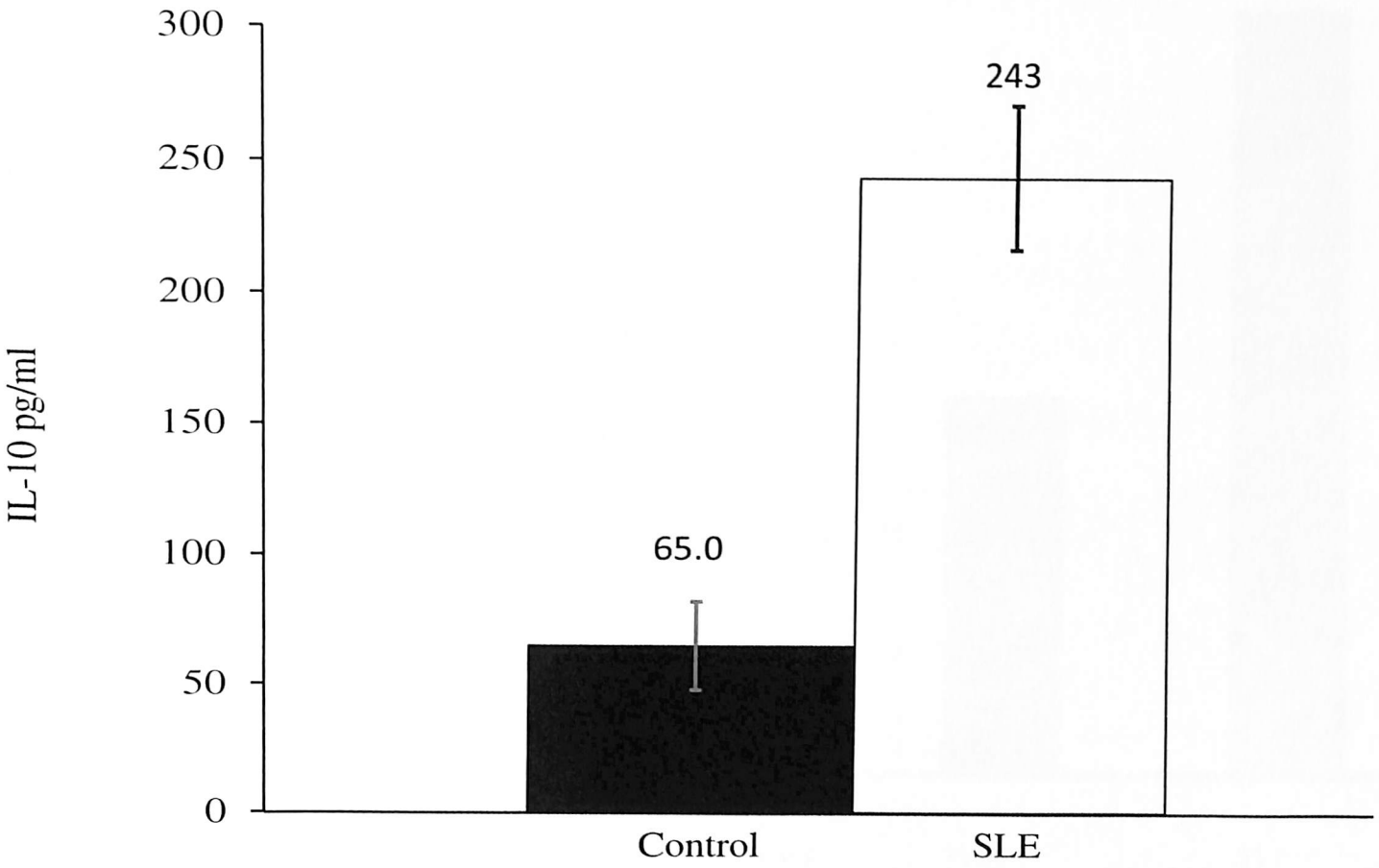

Figure 5: IL-10 Secretion by T-Cells. Media collected from T-cell cultures of Control and SLE mice. T-cell cultures were incubated with CD3/CD28 Dynabeads and IL-2 to stimulate baseline rate of IL-10 secretion, and assayed for IL-10 levels 48 hours after plating one million cells. T-cells collected from SLE mice secreted approximately 243 pg/ml of IL-10, while T-cells from control mice secreted 65 pg/ml.

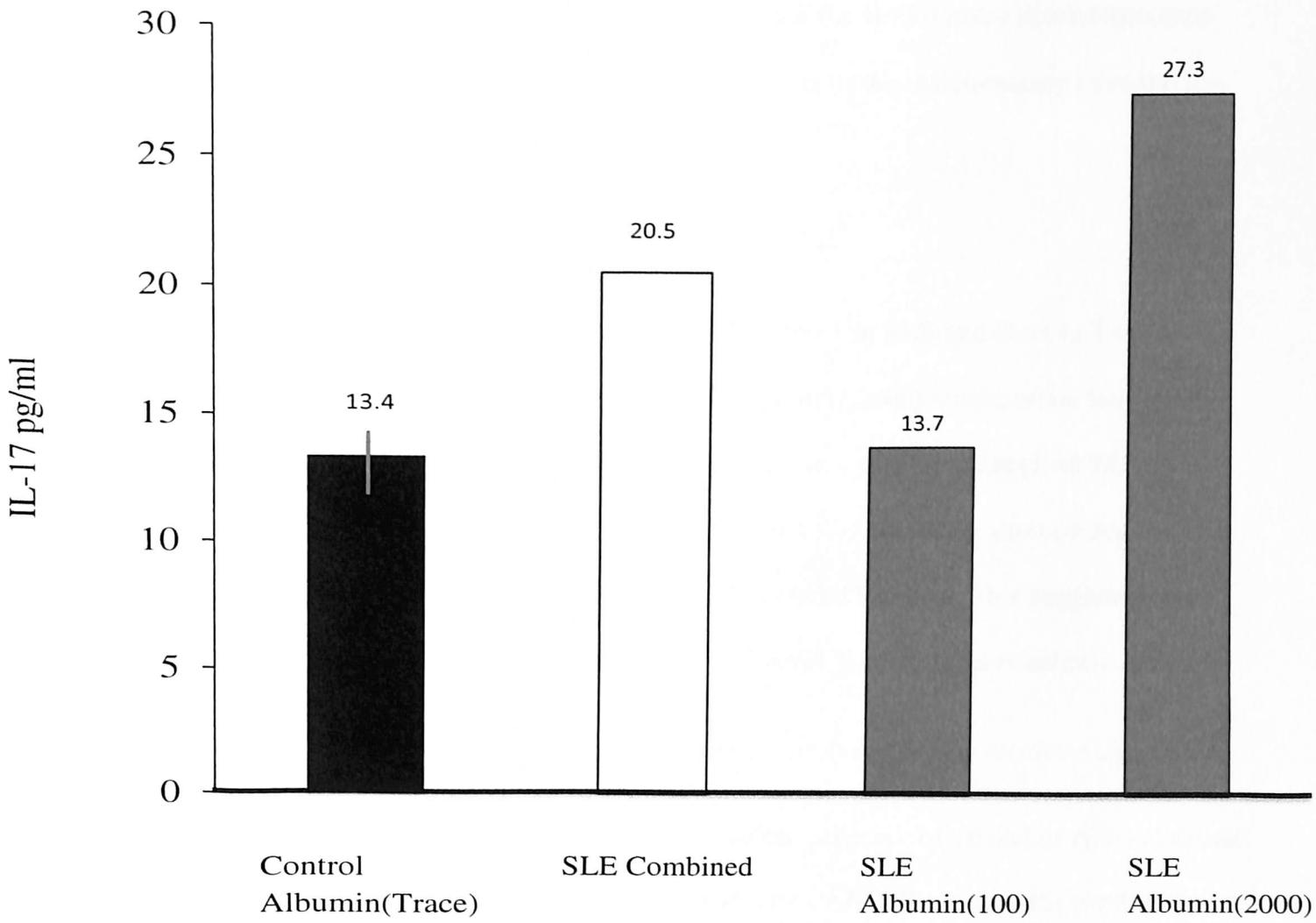

Figure 6: IL-17 Secretion by T-Cells. Media collected from Control and SLE mice' T-cell cultures were used to measure IL-17 secretion at baseline conditions in pg/ml. Albumin levels were measured in the urine pre experiment. Control mice had trace amounts of albumin, one SLE mouse had albumin levels of 100, and the other SLE mouse had Albumin levels of 2000. T-cells from control mice and SLE mice had average IL-17 secretion levels of 13.4 and 20.5 pg/ml respectively. The SLE mouse with 100 Albumin had secretion levels of 13.7, while the SLE mouse with high Albumin had secretion levels of 27.3 pg/ml.

always produce more IL-17 than Control. Yet, when the autoimmune disease becomes more severe, the SLE T-cells may be producing more of the inflammatory cytokine IL-17.

IFN- γ secretion is greater for SLE then Control T-cells.

Figure 7 compares the secretion of IFN- γ between SLE and Control T-cells at baseline conditions. The SLE mice had severe albumin (2000) levels, while the control mice had minor (trace).The media from the cell cultures was run through an ELISPOT assay, which forms spots based on the amount of IFN- γ. The assay showed that the SLE group created 156 spots while the Control group created 98 spots. This suggested that SLE T-cells were producing more IFN- γ then Control T-cells under baseline conditions.

SLE and Control T-cells in the presence of ANG-II increase IFN- γ secretion equally.

SLE and Control T-cells were cultured in the presence of 10 uM of ANG-II to see the effects on IFN- γ secretion. Figure 8 indicates that ANG-II increase the production of IFN- γ by T-cells in both groups. In the Control group the number of spots produced in the ELISPOT assay by IFN- γ increased from 98 spots without ANG-II to 192 spots with ANG-II (196% of control). Similarly in the SLE group, the number of spots jumped from 156 without ANG-II to 269 with ANG-II (172% of control). ANG-II did cause an increase in IFN- γ production, but did not cause any difference in the production of IFN- γ between the two groups.

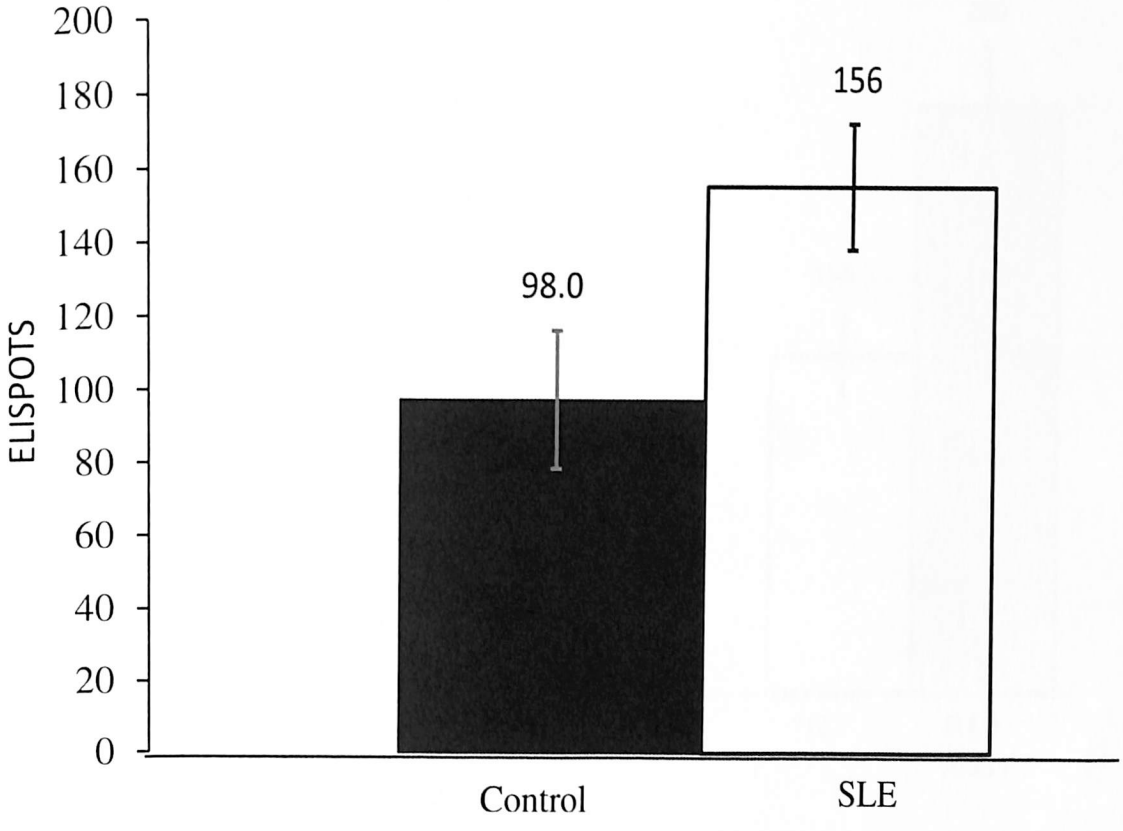

Figure 7: IFN- γ Secretion by T-Cells. Media collected from Control and SLE mice' T-cell cultures were used to measure IFN- γ secretion at baseline conditions by the formation of spots. T-cells collected from SLE mice created more IFN- γ based spots (156) than T-cells from control mice (98).

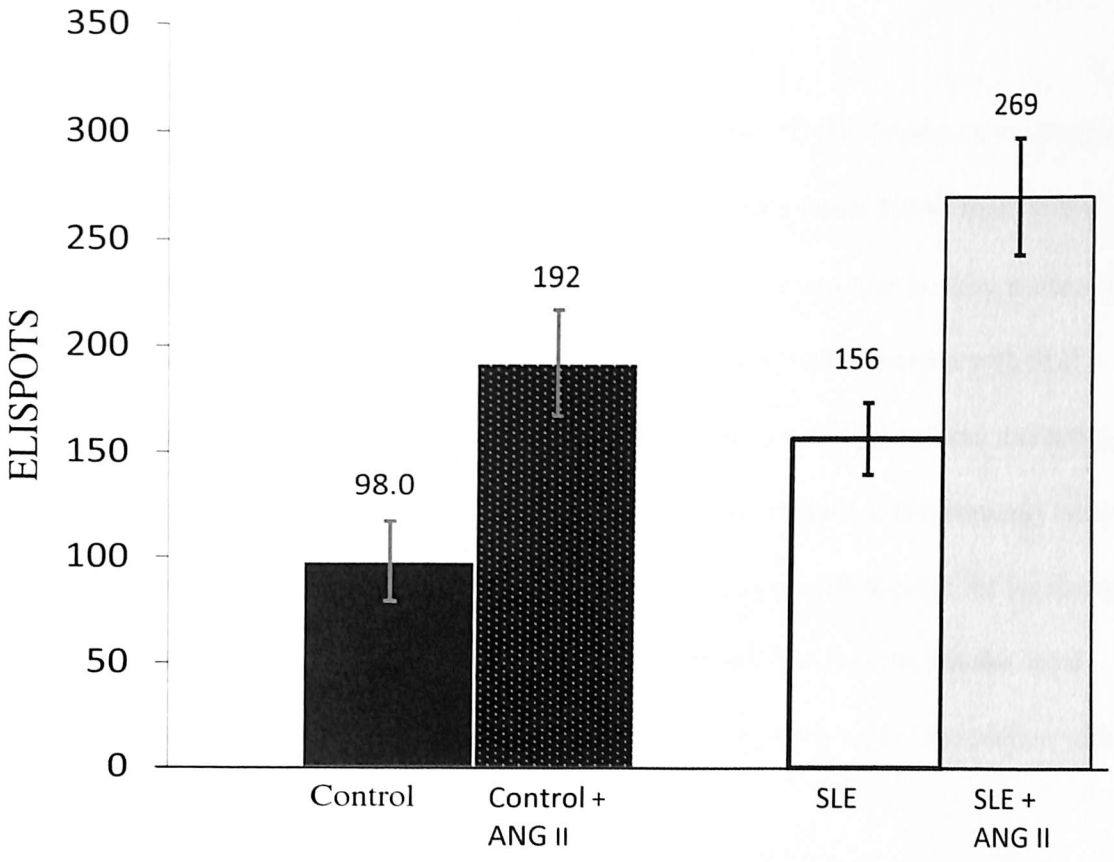

Figure 8: IFN- γ Secretion by T-Cells in the Presence of ANG II. T-cell media collected from Control and SLE mice were cultured in the presence or absence of ANG II. Media collected from the four different cell cultures were used to measure IFN- γ secretion by the formation of spots. ANG II caused more IFN- γ based spots to appear in the Control (+94), and SLE group (+114). There was only a minor difference in the IFN- γ based spots production between the two groups in the presence of ANG II.

Discussion

There is currently no cure for SLE, and patients with the disease must struggle their entire lives to maintain health. It has been well documented that women with SLE are at a much greater risk of dying from a cardiac related event then healthy women their age (5). The main risk factor for developing cardiac problems in women with SLE is hypertension, but the mechanism that leads to hypertension in SLE remains unknown. Since the kidneys are the long-term regulators of blood pressure, it is commonly believed that kidney damage is involved. SLE nephritis presents a plausible cause for the damage, but not all patients with SLE develop nephritis. Instead it has been shown that renal dysfunction, such as decreased GFR and water retention, has a higher correlation with hypertension than nephritis (12).

Changes in the forces that oversee the activity of the nephrons, RVR and RBF, preclude changes in renal function. Yet, these mechanics are tightly regulated in the kidneys, and it takes direct damage to the renal tissue to alter their controls (8). In SLE there is constant damage to tissues, as immune cells mistakenly identify and attack the body. This autoimmunity directly causes inflammation, and it is well known that the immune cells often lead to damage in the kidneys (1). Inflammation is a complex process that requires inflammatory cytokines to communicate between cells, and the levels of these cytokines dictate the severity of the inflammation caused tissue damage. This study focused on the levels of the inflammatory cytokines (IL-10, IL-17, IFN- γ), and proposed that the mechanism for SLE hypertension is centralized around the production of cytokines by T-cells.

To further test the role of inflammatory cytokines in the mechanism for SLE hypertension, the first step was to analyze their rate of secretion by T-cells isolated from Control and SLE mice. We predicted that there would be greater quantities of these cytokines being secreted by SLE T-cells, which would mean greater recruitment and activation of immune cells, potentially to the kidneys, and therefore more kidney damage. The NZBWF1 mouse model was a great tool to study SLE hypertension, since it mimicked the human disease, especially the increased MAP (Figure 4). The isolated T-cells from the mice were easy to culture, and after incubation provided great insight to the circulating levels of the different inflammatory cytokines.

The results illustrated that SLE T-cells were secreting IL-10 at a greater level then normal T-cells (Figure 5). Knowing that IL-10 is an anti-inflammatory cytokine makes this result seem contradictory, but the role of IL-10 is more complex in an autoimmune condition. While, it does act to reduce the number of T-cells in the circulation, it also stimulates B-cells to produce antibodies (21). This is detrimental in an autoimmune disease, because the increased numbers of antibodies continuously mistake the host tissue as foreign threats. Ultimately, more and more immune cells are recruited to the site of the antibodies, and the inflammation grows. In the kidneys of SLE patients, IL-10 may be stimulating the inflammation, and therefore degrading the kidney function. Results from this experiment showed that IL-10 production by SLE T-cells is almost 400% of Control T-cells production. The high prevalence and autoimmune consequences of this cytokine make it a potential player in the mechanism of SLE hypertension.

The pro-inflammatory cytokine IL-17 is known to recruit other inflammatory cells and promote inflammation (19). It was hypothesized that SLE T-cells would secrete this

cytokine at higher levels, but the results of the SLE group were variable. There was a major disparity between the IL-17 production levels of the two SLE mice, which correlated with major differences in their albumin levels. Albumin in the urine is a great indicator of how much damage has occurred in the kidneys, since the protein is usually too large to be filtered through the glomerulus (9). SLE is an unpredictable disease because the symptoms do not all appear simultaneously. Usually in the NZBWF1 mouse model the disease begins to manifest around thirty-six weeks, and all the mice develop severe symptoms soon after. The mice in this experiment were sacrificed exactly at thirty-six weeks, and it seems that one of them had not yet fully developed SLE. That mouse had only slight albumin in its urine, suggesting that it was beginning to develop kidney damage, but at the moment was fairly similar to the Control mice (albumin trace). The similar level of the pro-inflammatory IL-17 secretion by these two groups is consistent with the lack of kidney damage, and suggests that this cytokine contributes to inflammation in the kidneys. The other SLE mouse with severe albumin had T-cells that were producing IL-17 at almost 200% the rate of the Control. It is possible that the higher levels of IL-17 were causing more inflammation in the kidneys, and therefore more kidney damage in the SLE mouse with severe albumin. This suggests a possible role for IL-17 secretion in the mechanism for SLE hypertension.

Similar to IL-17, IFN- γ is also a cytokine known for promoting inflammation. Therefore, we predicted that SLE T-cells would secrete more of the cytokine than Control T-cells. The results indicated that at baseline conditions SLE T-cells were producing IFN- γ at about 150% that of Control production (Figure 7). The higher levels of the inflammatory cytokine suggest more inflammation occurring in the kidneys of the SLE

mice, and hence more renal dysfunction. Similar to the inflammatory cytokines IL-10 and IL-17, the results support the role of IFN- γ in promoting SLE hypertension through inflammation in the kidneys.

SLE T-cells secreted all three of the cytokines studied in this experiment at higher levels than Control T-cells, and all three fit in the proposed mechanism for SLE hypertension. Yet, it remained unclear what stimulated the T-cells to increase production, beyond the effects of IL-2 (which is normally present in vitro). The potential stimulus studied in this experiment was ANG II, since the hormone is well known to increase blood pressure (8). Previous experiments have shown that in some mouse models of hypertension, ANG II only promotes hypertension and renal dysfunction if immune cells are present (26). There is no concise evidence that ANG II levels are higher in patients with SLE. This lead to the hypothesis that ANG II was promoting hypertension by increasing inflammatory cytokine production, and therefore inflammation in the kidneys. Due to a lack of time and resources only the effects of ANG II on the cytokine IFN- γ were studied. Results showed that ANG II increased IFN- γ production about a 100% in both the SLE and Control group of T-cells (Figure 8). It had was hypothesized that SLE T-cells would see a sharper increase in IFN- γ production then control T-cells in the presence of ANG II. This would have supported the role of ANG II as the stimulus for SLE T-cells activation, but ANG II caused an equal increase in production in the groups. The results indicate that ANG II may be promoting inflammation in the kidneys as a means to promote hypertension. Yet, in the case of SLE there was no reason to suspect that ANG II is the trigger that causes the high levels of inflammatory cytokine production.

Ultimately the study supported the role of inflammatory cytokines in the development of renal damage. The results showed that three cytokines are being produced at greater levels by SLE T-cells. Knowing the chronic inflammation in SLE, the high prevalence of hypertension in SLE patients, and the previous studies that support the role of inflammation in hypertension, it is important to further study inflammatory cytokines in the development of SLE hypertension. It needs to be discovered what causes the T-cells in SLE to activate and promote inflammation. ANG II did increase the production of cytokines, but this increase was not specific to just SLE. Since SLE patients are at such a higher risk for developing hypertension, there might be a stimulus that specifically promotes SLE T-cells to activate. Since SLE is such a gender specific disorder, a potential candidate may be estrogen.

For most people the risk of a cardiac event can be prevented with simple things like exercise and diet. Yet, for women with SLE the risk of a cardiac event is present regardless of personal choices on fitness, and greatly increases their risk for mortality. The modern medication for these patients is focused on only mellowing the symptoms of hypertension, since the cause is not yet understood. More research on the inflammatory mechanisms that promote hypertension, would improve the prognosis for not only SLE patients, but patients of several autoimmune diseases and hypertension in general.

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