

THE INTERPLAY BETWEEN DIET, METABOLISM AND AUTOIMMUNITY IN THE B6.SLE
MOUSE MODEL OF SYSTEMIC LUPUS ERYTHEMATOSUS

By

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CHAPTER I

INTRODUCTION

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of auto-antibodies against self-antigens such as double-stranded DNA and phospholipids. Clinical complications of SLE include end-organ diseases such as glomerulonephritis, infection, vasculitis, arthritis, skin disorders, and neurological disease. Causes of SLE mortality can be clustered into two main groups: early mortality and late mortality (24, 61, 214). The early mortality group includes glomerulonephritis, infection and neurological disease, whereas the late mortality group is mainly composed of cardiovascular disease (CVD). Management of SLE complications has improved over the last several decades, causing a shift in mortality burden from early causes to late causes.

CVD represents a large component of late disease burden in SLE patients (95). In one study, nearly 40% of SLE patients were found to have carotid atherosclerotic plaques as compared to 15% of matched control subjects (181). Another study showed that 31% of lupus patients had coronary artery calcification whereas only 9% of controls showed calcification (119). The overall risk of CVD in females with lupus is five-fold greater than that of control subjects and this risk can be fifty times greater in some age groups (24, 119).

Although it is clear that SLE patients suffer disproportionately from CVD, the causes of this disparity have not been elucidated. Various studies have shown a higher prevalence of traditional CVD risk factors such as hyperlipidemia or hypertension and non-traditional, SLE-

specific risk factors such as autoantibody production or corticosteroid therapy (24). One of these traditional risk factors, insulin resistance (IR), has higher prevalence in the SLE population (116) and may contribute to the high CVD burden in these patients. IR has been associated with CVD risk in many studies (168, 172, 241) and the contribution of insulin resistance to atherosclerosis in the context of SLE merits further study.

Although IR is increased in prevalence in the SLE population, the absolute number of IR-afflicted individuals in the general population is exponentially higher. This IR epidemic largely results from the increasing numbers of obese and overweight individuals in the developed world. The pathogenesis of many obesity comorbidities, including IR, is heavily influenced by systemic and tissue-specific inflammation. This inflammation arises from interactions between the metabolic and immune systems, and white adipose tissue (WAT) has emerged as a key mediator of this interaction. WAT, classically described as an inert energy storage depot, has recently been shown to play an active role in both metabolic homeostasis and immunity. These capabilities are conferred by the ability of adipocytes to secrete more than seventy-five adipokines (230), which include a variety of chemokines, cytokines and hormones that can affect appetite, recruit leukocytes to WAT, and instigate systemic metabolic disruption (149, 179, 195). The recruited T cells, B cells and macrophages work in tandem with adipocytes to give rise to the metabolic syndrome.

Recent findings have provided circumstantial evidence that obesity-related IR and SLE-related IR share common pathogenic mechanisms. Although the role of the adaptive immune system—specifically auto-antibody production—has long been known to be involved in the development of SLE, B cells have only recently been shown to act as central regulators of IR (224). Specifically, B cells produced pathogenic IgG antibodies in the context of obesity which

are sufficient to confer insulin resistance in passively immunized recipient mice. Therefore, B lymphocytes comprise a potential link between the pathogenesis of IR in SLE and obesity.

There are limited data regarding metabolic syndrome in mouse models of SLE. In the only other study of metabolic syndrome in an SLE model of which we are aware, Ryan et al. (185) have shown that the lupus-prone (NZB/W)F1 strain has increased adiposity, increased macrophage infiltration into adipose tissue, and increased glucose intolerance, but the contributions of the adaptive immune system to this metabolic phenotype were not examined. Therefore, the role of lymphocytes in the pathogenesis of insulin resistance in the context of SLE is not known and merits further study. Furthermore, there are no reports on metabolic syndrome in other mouse models of SLE.

Chapter two of this thesis describes efforts to characterize the development of glucose homeostasis in the lupus-prone B6.SLE.1.2.3 strain of mice. We show that B6.SLE mice have significantly worsened glucose tolerance and adipose tissue insulin resistance than B6 controls. This B6.SLE glucose intolerance occurs in the presence of a diabetogenic B cell environment which is characterized by the presence of an increased number of IgG-producing B cells and IgG depositions in the white adipose tissue as well as higher levels of circulating IgG. Most interestingly, this strain of mice develops glucose intolerance and adipose tissue insulin resistance in an adiposity-independent manner, indicating that this strain may be a valid model for insulin resistance in the SLE patient population. These studies provide data that elucidate the pathogenesis of insulin resistance in SLE patients and may provide greater insight into the development of insulin resistance in the obese population.

Chapter three shows that high-fat diet feeding does not significantly worsen glomerulonephritis or kidney function in B6.SLE mice. Although this finding is in conflict with

earlier studies of SLE models, it is possible that changes in study design could reveal dietary factors that exacerbate or ameliorate disease in the B6.SLE mouse model. Chapter four compares our results to those of other studies in SLE model models and SLE patients and proposes a mechanism for the pathogenesis of glucose intolerance and insulin resistance in the B6.SLE mouse model.

Systemic Lupus Erythematosus

Early History of Systemic Lupus Erythematosus

The term “lupus” dates to the 10th century A.D. and referenced the similarity of the cutaneous lesions to wolf bites (118, 192). The diagnosis of lupus was refined over subsequent centuries until Laurent Biett and Louis Cazenave generated the term “lupus erythematosus” in the early 1800s and subdivided the condition into three subtypes distinguishable by the nature of the cutaneous lesions that were present: tubercular lupus, ulcerative lupus and hypertrophic lupus (216). The diagnosis of lupus erythematosus was specifically reserved for the cutaneous manifestations of the disease until Moriz Kaposi described concomitant visceral complications of lupus erythematosus in an 1872 article (192). He noted that lupus patients were often found to have lymphadenopathy, joint swelling and pain, and anemia. His reports set the path for a more holistic understanding of the symptoms of the disease. Kaposi’s writings, along with a series of lupus case reports written by Sir William Osler (154) helped to popularize the concept of lupus erythematosus as a systemic disease involving diffuse organ systems.

The first conclusive evidence that immunoglobulins were involved in the pathogenesis of SLE was gathered by Malcom Hargraves in 1948, who found that SLE patient serum could cause granulocytes—then named lupus erythematosus cells, or LE cells—to react with the nuclei of

bone marrow cells in normal patients (118). The target of the serum factor eliciting this LE cell response was identified as nuclear material by Miescher and Fauconnet who found that adsorption of SLE patient serum with nuclear lysates prevented the LE cell cytotoxic response (129). This line of research led George Friou to discover in 1958 that the LE factor was immunoglobulin and that one of its targets was the DNA:Histone complex (66, 67). Subsequent studies by other research groups showed that SLE patients had antibodies that are specific for a wide variety of self antigens. The finding that antibodies play a pivotal role in the pathogenesis of SLE paved the way for the hallmark screening test for lupus, the anti-nuclear (ANA) antibody test, a screen for autoantibodies including anti-dsDNA, anti-histone, anti-Ro, anti-Sm, anti-cardiolipin and anti-C1q (171). Furthermore, the findings of Hargraves, Friou, Miescher, Fauconnet and other research groups provided critical early cues regarding the pathogenesis of SLE and initiated the modern era in the field of SLE.

Modern Understanding of the Pathogenesis of SLE

SLE is now recognized as systemic disease in which the immune system produces auto-antibodies against various tissue antigens. These antibodies give rise to an inflammatory cascade involving the complement system, innate immune cells and T cells. As recognized by Osler and Kaposi, SLE can manifest itself in a variety of organ systems including the kidneys, the liver, the integumentary system, the musculoskeletal system, the cardiovascular system and the central nervous system. Each of these manifestations share common underlying mechanisms in which antibodies cause dysfunction and damage directly or indirectly through engagement of the innate and adaptive immune systems.

One of the most common manifestations of SLE is lupus nephritis. Nephritis can arise when antibody:nuclear protein complexes deposit on the glomerular basement membrane and

cause a type III hypersensitivity reaction (171). These immune complexes elicit activation of the complement cascade—mainly via the classical pathway—which can cause direct damage to the nephron through membrane attack complexes and indirectly through recruitment of granulocytes, NK cells and macrophages to the basement membrane. Additionally, autoantibodies against nuclear antigens like dsDNA and chromatin can cross-react with kidney antigens causing activation of the complement cascade and engagement of the innate immune system via the Fc receptor (13). These mechanisms are broadly applicable to other tissues targeted in SLE.

Less commonly, antibodies can have direct effects by acting as ligands for cell surface receptors. An example of this phenomenon is neuropsychiatric lupus secondary to autoantibodies against the NMDA receptor. These antibodies can function as antagonists or agonists of the NMDA receptor, eliciting depression, cognitive impairment and/or psychosis (105). Similarly, antibodies against insulin receptors can act as agonists of the insulin receptor, causing severe hypoglycemia, or antagonists, causing a subtype of insulin resistance classified as type B insulin resistance (158).

The production of autoantibodies in SLE depends on the failure of several checkpoints that normally protect against autoimmunity. Firstly, clearance of apoptotic material in lupus patients and mouse models of lupus has been shown to be impaired, which permits exposure of nuclear antigens to the immune system (146). Secondly, several major histocompatibility regions, including HLA-A1, HLA-DR2 and HLA-DR3 are associated with lupus and may contribute to more efficient presentation of self-antigens (193). Negative selection of autoreactive B cells can also be impaired in the setting of lupus. Autoreactive B cells can be eliminated at several checkpoints: receptor editing, anergy, clonal deletion, and light chain editing in pre-B cells and immature B cells (17). Overactive light chain editing has been

observed in humans and mouse models of lupus and has been associated with the production of autoreactive B cells (17, 113). Furthermore, the over- or under-expression of various cytokines such as B cell activating factor (BAFF) can contribute to survival of autoreactive B cells (35, 190). Finally, mutations in several intracellular proteins and co-receptors related to negative selection can allow autoreactive B cells to mature and produce auto-antibodies that contribute to SLE pathogenesis. For example, the expression of Ly108.2 isoform of the Ly108 co-receptor has been shown to be carried in the lupus susceptibility region of a mouse model of SLE and contributes to the survival of autoreactive B cells through interference with the apoptotic pathway and altered receptor editing (218).

Dysfunction has also been observed in T cells from SLE patients and mouse models of SLE. One of the most striking changes in the T cell population in the context of SLE is a deficiency in the ξ chain of the TCR complex. In one study, SLE patients had reduced or absent ξ chain, a TCR complex component which bears phosphorylation sites necessary for transduction of TCR signals (111). In a follow-up study, the same research group found that the ξ chain had been replaced by Fc ϵ RI γ chain, which is normally a component of the IgE receptor (55). This substitution causes more intense signaling through the TCR, possibly through the utilization of Syk kinase in lieu of the canonical Zap-70 kinase (55).

The receptors, co-activator molecules, and intracellular machinery necessary for activation of T cells reside in lipid rafts. Lipid rafts in SLE patients have been shown to vary in composition and location, and appear to be primed to respond to stimulus through the TCR. In one study (92) SLE T cells were shown to have increased CD45 and activated LCK occupancy in lipid rafts and accelerated recruitment of the lipid raft to the immunological synapse. Furthermore, the expression of c-Cbl, a ubiquitin ligase involved in the degradation of LCK, was

shown to be reduced in SLE T cells, providing a potential mechanism for the deranged LCK activity in SLE lipid rafts.

Co-stimulators and adhesion molecules involved in the activation, migration or suppression have been shown to be altered on the T cells of SLE-inflicted individuals. For example, the induction of CTLA-4 is impaired in activated T cells from SLE patients (112) and polymorphisms in the CTLA-4 gene are associated with SLE (213). Since this molecule serves to dampen the activity of activated T cells, its deficiency could contribute to the hyper-responsiveness of T cells in the context of SLE and serves as a potential drug target (130). The CD40L costimulatory molecule is expressed on T cells and activates B cells to produce immunoglobulins by signaling through CD40. CD40L expression has been shown to be increased in resting and activated T cells in SLE patients (49, 103). Finally, the expression of adhesion molecules that facilitate the migration of pathogenic T cells has also been shown to be increased in SLE patients. In one study, the severity of SLE symptoms was shown to correlate with the level of CD44 expression on patient T cells (59). CD44 is a lipid-raft resident protein that is important for the migration of T cells into inflamed tissue.

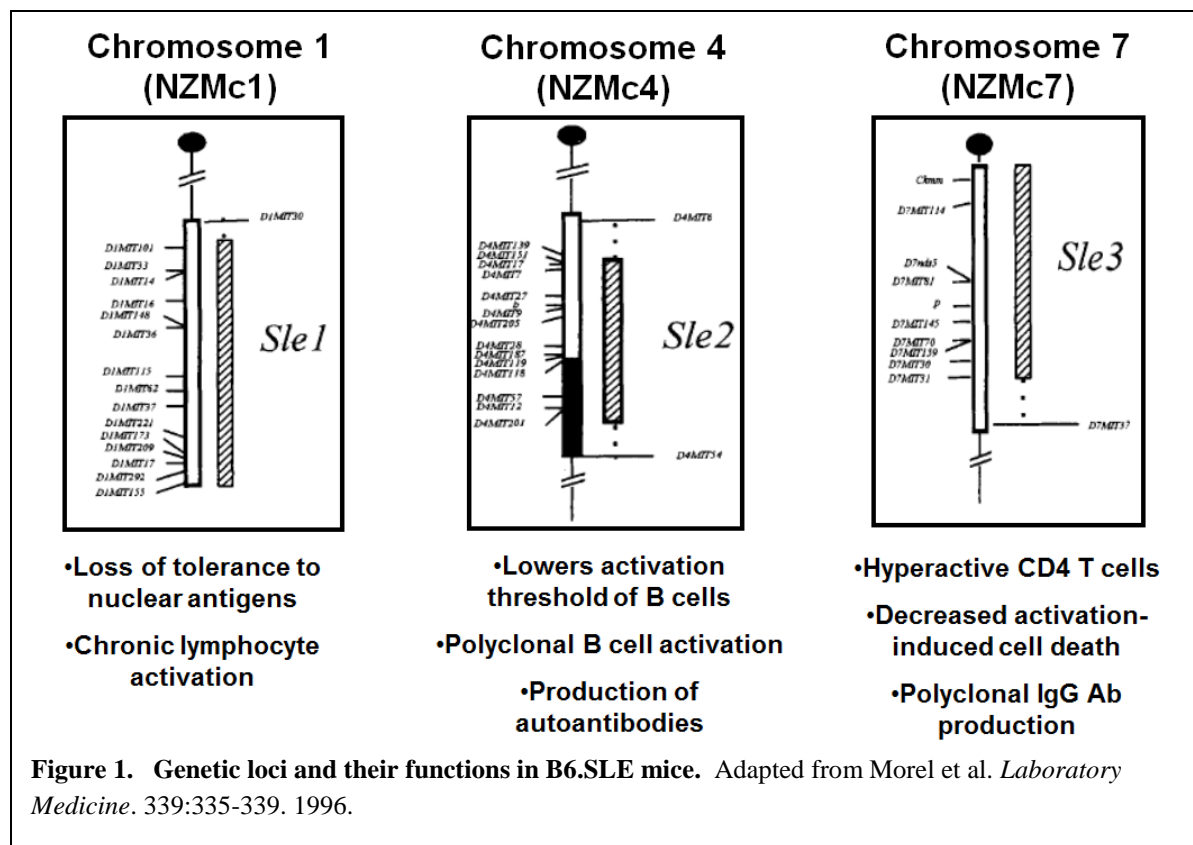
IL-2 is, a cytokine expressed by T cells, serves in an autocrine fashion to maintain activation and proliferation. Gene expression for the receptor and the cytokine is upregulated in response to ligation of the TCR. IL-2 production is impaired in activated T cells from SLE patients (45). Although lower IL-2 production would be expected to reduce the immune response, IL-2 deficient mice have been shown to develop autoimmune disease (187). This paradoxical response could be explained by the absolute necessity of IL-2 for proper T regulatory cell development (150). Deficiencies in the T regulatory cell population could counteract the effects of impaired T cell proliferation and activity.

Subpopulations of T lymphocytes may protect against or exacerbate SLE. As described above, T regulatory cells protect against excessive inflammation and reactivity against self-antigens. T regulatory cells have been shown to be impaired in SLE patients and mouse models of SLE. For example numbers and functions of T regulatory cells have been shown to be reduced in the setting of SLE (18, 115, 145, 157).

Th17 cells are a pro-inflammatory subset of CD4+ T cells which have been linked to the pathogenesis of several autoimmune diseases including rheumatoid arthritis, Crohn's disease and SLE. As indicated by their name, Th17 cells secrete IL-17 which can contribute to the activation and survival of pathogenic leukocytes (71). Circulating IL-17 has been shown to be increased in SLE patient serum (228, 229) and Th17 cells have been shown to infiltrate the kidneys in the setting of lupus nephritis in both humans and mouse models (7, 237).

Mouse Models of SLE

The NZB/W F1 mouse model of SLE is one of the oldest and most widely used models of spontaneous SLE. In this model, lupus prone mice are generated through the mating of New Zealand Black mice with New Zealand White mice. The F1 generation of this cross has a fully-penetrant phenotype characterized by the production of autoreactive IgG and glomerulonephritis (160). Many other lupus-susceptible strains have been generated from the NZB/W F1 strain through backcrossing with parental mice. One of these strains—the NZM2410 inbred strain—became the basis of extensive genetic testing for the susceptibility regions that predispose this family of SLE mouse models to the production of autoantibodies. In a study published in 1994, *Morel et. al* backcrossed NZM2410 mice on the C57BL/6 background and used genetic interval mapping to identify regions that created susceptibility for glomerulonephritis (139). These regions, named *Sle1*, *Sle2*, *Sle3*, and *H-2* are mapped to chromosomes 1, 4, 7 and 17,



respectively. In a later study, the same group used microsatellite marker mapping to backcross the *Sle1*, *Sle2*, *Sle3*, and *H-2* lupus susceptibility regions on to the C57BL/6 background to create the B6.Sle.1.2.3 strain, heretofore referred to as B6.SLE mice (141). Subsequent genetic dissection of the susceptibility regions revealed the specific defects carried by each region (Figure 1). Mice bearing the *Sle1* susceptibility region lost tolerance to anti-chromatin antibodies, which was attributed to an increase in histone-reactive T cells (133). *Sle2* was shown to elicit a hyperactive B cell phenotype which extends and amplifies autoimmunity (134). Finally, mice bearing *Sle3* were shown to have accumulation of activated CD4⁺ T cells, increased T cell proliferation in response to stimulation and reduced apoptosis (135). Collectively, these susceptibility regions confer a fully penetrant lupus phenotype characterized by the production of autoantibodies against nuclear antigens and fulminant glomerulonephritis. Although human females are more likely to develop lupus, B6.SLE show nearly equal

penetrance of glomerulonephritis between genders (139). Additionally, *Sle1* is syntenic with regions of human chromosome 1 that have been shown to be linked to development of SLE in humans (60).

Other spontaneous mouse models of SLE include the MRL/lpr and BXSB/Yaa strains. The MRL/lpr strain carries a defective Fas apoptosis signaling molecule which facilitates survival of autoreactive T cells during negative selection (124). MRL/lpr are one of the only spontaneous models that develop arthritis and skin lesions (73), and may be particularly suited for the study of neuropsychiatric lupus (76). The BXSB/Yaa strain carries lupus susceptibility genes on the y chromosome (the y-linked autoimmune accelerator) which were duplicated secondary to a translocation from the x chromosome (160, 164). This translocation predisposes male mice from this strain to developing a lupus-like phenotype consisting of the production of auto-antibodies against nuclear antigens and immune-complex-mediated glomerulonephritis.

The most commonly used model of inducible lupus is the pristane mouse model. Pristane, also known by its chemical name tetramethylpentadecane, is a hydrocarbon that was found to induce a lupus-like syndrome when injected intraperitoneally. The lupus phenotype in these mice is characterized by the production of auto-antibodies against nuclear antigens, arthritis and serositis and glomerulonephritis secondary to the deposition of immune complex on the basement membrane (175, 191).

Lupus Therapy

When Osler published his widely cited series of case reports on suspected SLE patients at the turn of the 20th century, the available therapy for SLE was rudimentary and mainly supportive (154). This was summarized in his 1903 text, in which he wrote:

The very chronic cases [of SLE] with recurring colic for years may resist all measures. Alterative courses of gray powder, with careful dieting, may be helpful. With angioneurotic edema nitroglycerin in full doses may be tried...The chief danger is from the kidneys and in so frequently presenting this subject I have hoped to impress upon my colleagues the importance of recognizing this form of nephritis, and of taking early precautions to prevent its progress, against which I think protracted rest in bed and a milk diet are the best means at our disposal.

The pharmacological armamentarium for SLE treatment has remained limited since those words were written by Osler 100 years ago. Perhaps one of the largest advances in the treatment of SLE was the discovery and clinical utilization of corticosteroids in the first half of the 20th century, although corticosteroids weren't approved for use in SLE by the FDA until 1955 (118). Additional major FDA-approved SLE drugs were aspirin (approved in 1948) and the anti-malarial agent chloroquine (approved in 1955) (131). Other immunosuppressants have been used off label in the treatment of SLE, including mycophenolate mofetil, cyclophosphamide, and rituximab (211). However, many of these therapies—especially the immunosuppressants—carry significant side effects and may not be suitable for long-term treatment regimens. Belimumab, a monoclonal antibody against soluble B cell activating factor was the next drug approved by the FDA in 2011, over 50 years after the approval of corticosteroids and chloroquine. However belimumab was not tested in African Americans or in patients with lupus nephritis; therefore, its utility in the treatment of these large subsets of lupus patients is unknown.

Metabolic Syndrome

Overview of Metabolic Syndrome and Inflammation

The metabolic syndrome consists of several inter-related disorders, including the “deadly quartet” of truncal obesity, insulin resistance, hypertension, and dyslipidemia (8, 18). Insulin resistance, a key component of type 2 diabetes mellitus (T2DM), is thought to be caused by chronic inflammatory processes involving complex interactions between cells of the metabolic and immune systems. The link between inflammation and diabetes was first shown in the late 19th century when W. Ebstein treated his T2DM patients with large doses of aspirin, a non-steroidal anti-inflammatory drug (46).

More contemporary studies have yielded crucial information linking diabetes and inflammation. Fat tissue has been shown to be a main focus of the chronic inflammation that gives rise to insulin resistance and T2DM. Examination of fat tissue has revealed an accumulation of adipose tissue macrophages (ATMs) in obese mice (59), and blocking the accumulation of these cells attenuates insulin resistance (58). ATMs have been shown to be key mediators of inflammation and insulin resistance in the setting of obesity (58, 59). This field has focused on the opposing roles of pro-inflammatory classically-activated (M1) macrophages and anti-inflammatory alternatively-activated (M2) macrophages in adipose tissue inflammation. The development of M1 macrophages is induced by bacterial products such as LPS and the T-helper type 1 (T_H1) cytokine IFN- γ , whereas M2 macrophages are induced by T-helper type 2 (T_H2) cytokines such as IL-4 and IL-13 (37). M1 macrophages produce proinflammatory mediators including TNF α , IL-6, IL-12, nitric oxide and reactive oxygen species and are thought to play a role in combating bacterial infections. Conversely, M2 macrophages produce the anti-inflammatory cytokine IL-10 (3) and are thought to play a role in tissue remodeling. Insulin

resistance is ameliorated in both transgenic mice deficient in M1 ATMs and mice treated with pharmacological agents that shift ATMs to the M2 phenotype (17, 38). Additionally, similar manipulations instigate M2 deviation in hepatic Kupffer cells and improve whole body insulin resistance. Both macrophages and adipocytes appear to serve as sources of the inflammatory cytokines TNF α and IL-6, which are abundant in the adipose tissue of obese individuals (60).

More recent studies have implicated the adaptive immune system in the pathogenesis of insulin resistance. CD8⁺ T cells exacerbate and T regulatory cells ameliorate insulin resistance in the context of obesity (62, 151, 225), and B cells have been shown to exacerbate insulin resistance through the production of pathogenic immunoglobulin (224).

Several mechanisms of obesity-induced immunoactivation have been proposed. The endoplasmic reticulum (ER) acts a sensor of metabolic stress which can promote the inflammatory state by influencing NF κ B and JNK signaling (15, 40), and oxidative stress induces both insulin resistance and the secretion of pro-inflammatory cytokines in adipocytes (12, 51). Additionally, members of the peroxisome-proliferator activated receptor (PPAR) family modulate immune system function by acting as sensors of fatty acids (44). Interestingly, pattern recognition receptors on adipocytes, macrophages and dendritic cells appear to play a role in diet-induced inflammation (26, 45, 65). Toll-like receptor 4 (TLR4), most commonly known as a sensor of bacterial lipopolysaccharide (LPS), has been shown to mediate the production of pro-inflammatory cytokines (45) and cyclooxygenase 2 (COX-2) (26) in response to saturated fatty acids. COX-2 is a key component of the prostaglandin biosynthesis pathway and is the target of an important class of anti-inflammatory drugs. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) have been found to suppress the effects of saturated fatty acids by blocking TLR4-mediated activation of macrophages, dendritic cells, and adipocytes (26, 45, 65).

The signals resulting from obesity-induced ER stress, oxidative stress, PPAR ligation, and TLR signaling contribute to a pro-inflammatory state in the affected cells, characterized by the secretion of inflammatory cytokines. These cytokines give rise to more inflammation within the adipose tissue, creating a “vicious cycle” of chronic inflammation. These signals directly contribute to insulin resistance by acting on the JNK pathway ultimately causing the inactivation of insulin receptor substrate 1 (IRS-1) which is a key mediator of insulin receptor signaling (43, 46).

Insulin sensitivity can also be affected through modulation of PPAR-gamma, a transcription factor that acts as a central regulator of adipocyte development and activity. The thiazolidinedione class of drugs—including rosiglitazone and troglitazone—has been efficacious in the treatment of type 2 diabetes in humans and was thought to act as classical agonists of PPAR-gamma. In turn, PPAR-gamma activity was hypothesized to exert its insulin-sensitizing effects by causing beneficial lipid storage in adipocytes (termed the “lipid steal” hypothesis) and by modulating the production of adipokines in adipocytes (207). More recent studies have indicated that obesity-induced inflammatory stimuli upregulate Cdk5 activity, which in turn mediates phosphorylation of PPAR-gamma at its serine 273 residue. This phosphorylation modulates the activity of PPAR-gamma, causing downstream effects which ultimately result in insulin resistance. Thiazolidinediones were found to prevent this phosphorylation, allowing PPAR-gamma to exert an adipocyte transcriptional program that protects against insulin resistance (36). Although thiazolidinediones are thought to act primarily on adipocytes, studies by Benoist et al. showed that they also promote the expansion of T regulatory cells in the adipose tissue (40). This expansion of immunosuppressive lymphocytes protects against obesity-induced inflammation and insulin resistance. Interestingly, PPAR-gamma agonists have also been shown

to ameliorate autoimmune disease in mouse models, including the multiple sclerosis model experimental autoimmune encephalomyelitis (102).

An additional factor that has been shown to play an important role in glucose homeostasis is the intestinal microbiota. The ground-breaking studies of Gordon et al. showed that obese mice have a unique intestinal bacterial profile relative to lean controls and that these bacteria can confer weight gain and glucose intolerance when transferred into gnotobiotic mice (50). These microbial changes were also shown to occur in humans. Studies of twins showed that mono- and dizygotic twins shared a core component of bacterial species, but that an obese twin had a less diverse and significantly different array of bacterial species than their lean counterpart twin (212). Furthermore, modulation of the microbiota with broad-spectrum antibiotics ameliorated glucose intolerance and insulin resistance in obese mice, perhaps by reducing levels of circulating LPS and inflammatory cytokines (30). The microbiota has also been shown to modulate pathogenesis of autoimmune disease in several mouse models. Rheumatoid arthritis-prone K/BxN and *Il1rn*^{-/-} mice do not develop disease in germ free conditions, but the introduction of segmented filamentous species or *Lactobacillus bifidus*, respectively, were able to induce disease in these models (1, 235). Similarly, the experimental autoimmune encephalomyelitis mouse model of multiple sclerosis develops less severe disease under sterile conditions. Conversely, segmented filamentous bacteria exacerbate disease through modulation of IL-17 producing Th17 cells (107).

Mouse Models of Metabolic Syndrome

The most widely utilized genetic mouse models of obesity are the *ob/ob* and *db/db* strains. The *ob/ob* strain resulted from a spontaneous mutation in a house mouse colony at the Jackson Laboratory and was published in a landmark article that described the discovery of some

“very plump young mice” during the summer of 1949 (89). The *ob/ob* mice had rapid weight gain relative to their controls, ultimately achieving body weights over two times greater than their littermates. Sixteen years later, the parental mice of the *db/db* strain were discovered in another mouse colony at the Jackson Laboratory (84). The phenotype of this new strain was indistinguishable from that of *ob/ob* strain and both strains demonstrate rapid and uncontrolled weight gain and hyperglycemia. The similarity of the phenotype was a source of curiosity for scientists in the obesity field and clues regarding the underlying mutations for each strain were provided by classic experiments completed by D.L. Coleman at the Jackson Laboratory (41). In his experiments, parabiosis was used to link the circulatory systems of *ob/ob* and *db/db* mice with each other and with control strains. In his discussion, Coleman wrote:

These observations provide further support for the conclusion that the obese [*ob/ob*] mouse has a functional satiety center and that it is capable of responding to satiety factor produced by normal as well as diabetes [*db/db*] partners. If these conclusions are correct, the identical obese-hyperglycemic syndromes produced by these two unrelated and separate genes would be explained. In one case, the target organ (satiety center) is defective while in the other the satiety factor is either not produced or is produced in insufficient quantities. Both mechanisms would cause over-eating and produce identical secondary changes in both obese and diabetes mice.

His conclusions aptly predicted the results of another landmark study by Zhang et. al conducted two decades later, in which the mutated gene from *ob/ob* was found to encode a secreted protein which later became known as leptin (243). Two years later, the leptin receptor was identified and was implicated as the defective gene in *db/db* mice (206) . Leptin has since been shown to be an adipocyte-secreted hormone that provides a satiety signal to receptors in the hypothalamic feeding center (65). In *db/db* mice, the leptin receptor is mutated and cannot transduce the satiety signal to feeding center in the hypothalamus. In contrast, *ob/ob* mice lack leptin and therefore cannot signal satiety to the hypothalamus. Both mutations result in the uncontrolled eating and weight gain seen in *db/db* and *ob/ob* mice. The obesity contributes to the severe insulin resistance and hyperglycemia seen in these models.

Another commonly-used model of mouse obesity is the diet-induced model (DIO). In this model, obesity is induced by feeding mice a diet high in kilocalorie percentages from fats which is meant to replicate the modern Western diet. The weight gain seen in these models is generally not as rapid or severe as seen as in *ob/ob* or *db/db* strains, but is perhaps more analogous to the amount of weight gain seen in humans eating a fatty diet. However, DIO mice do develop insulin resistance and other components of the metabolic syndrome. A chief advantage of this model—aside from its relatively greater clinical relevance—is the ability to induce obesity in any strain of mice needed for experiments.

Immunity and Nutrition: Immunity in the Setting of Starvation and Malnutrition

Although malnutrition has long been associated with increased risk of infection, the immunological mechanisms behind these observations were not described until Ranjit Chandra published a series of papers in the 1970s (31–34). Dr. Chandra and others showed that protein-energy malnourished (PEM) children had lymphoid tissue involution, impaired humoral and

cellular adaptive responses to antigens, and defective neutrophil function. These deficiencies led to impaired primary response to infection and attenuated response to poliovirus and measles vaccines (31). Studies from other groups showed that PEM rodents had reduced numbers of blood and bone marrow leukocytes, impaired leukocyte migration into areas of sterile inflammation and decreased LPS-stimulated production of TNF- α , IL-1 β , and IL-6 of peripheral blood leukocytes (19, 51, 147). Additionally, PEM rodents have higher mortality rates when challenged with influenza virus (178) and salmonella (159). Specific changes in the lymphocyte compartment have also been observed in both PEM mice and humans. Malnourished mice had significantly higher proportions of quiescent T cells in the blood and secondary lymphoid tissues than in well-nourished controls (232, 233). Furthermore, T cells in PEM rats had a significantly reduced ability to synthesize and secrete IL-2 and Ifn- γ (126). More recent studies have shown that PEM prevents maintenance and expansion of memory CD8⁺ T cells and memory B cells in mice (91, 121) and suppressed generation of effector T cells in malnourished children suffering from infection (152). Collectively, these PEM-associated immune deficiencies inhibit host defense against pathogens and prevent the generation of immunological memory after primary infections and immunizations.

Similar results have been observed in patients suffering from anorexia nervosa. There is a reduction in the numbers of circulating T cells in anorexic patients (120) and those that remain have lower Ifn- γ secretory capacity and reduced proliferation capacity (165). Additionally, there is a marked decrease of CD8⁺ memory cells in anorexic patients (166) which is accompanied by deficiencies in delayed type hypersensitivity (Type IV hypersensitivity) response in the most severe cases of anorexia (161). These results conflict with other studies which showed normal

generation of humoral and cellular immunity in anorectic patients (8, 75, 169), a discrepancy which may be explained by differences in patient group disease severity between studies.

Calorie restriction has been widely promoted as a way to increase longevity, but recent studies have called this into question. Two parallel calorie restriction studies conducted at the National Institute of Aging (NIA) and the University of Wisconsin demonstrated the importance of macronutrient and micronutrient dietary content in modulation of aging (42, 125). The University of Wisconsin study demonstrated increased longevity in rhesus monkeys fed 30% fewer calories from their respective baseline consumption versus controls fed the same diet ad libitum. The NIA study, however, showed no difference in longevity between mice fed 30% fewer calories than baseline consumption and control monkeys who were fed 100% of their baseline caloric consumption. The authors of the NIA study hypothesized that different dietary components between their study and the University of Wisconsin study accounted for the different study results. Namely, the Wisconsin study utilized a diet with ten times higher sucrose content than that used in the NIA study, whereas the diet used in the NIA study contained a significant amount of anti-oxidant flavonoids and omega-3 fatty acids from fish oil. Finally, all study monkeys were given supplementary vitamins and minerals whereas only the calorie restricted animals were given supplements in the Wisconsin studies.

There has been limited study of immune function in the context of calorie restriction. A six-month calorie restriction regimen in overweight humans increased the delayed-type hypersensitivity reaction and proliferation of T-cells in response to tuberculin and candida albicans antigens (2). Calorie restriction has been shown to prevent T cell senescence in rhesus monkeys (128), although T cell function can be negatively impacted or unaffected if calorie restriction is initiated in juvenile monkeys or “elderly” monkeys, respectively (127). Studies in

mouse models showed that calorie restriction prevented age-related thymic involution and maintained T cell function and the T cell receptor repertoire (238). Furthermore, calorie restriction ameliorates disease in rodent models of experimental autoimmune encephalitis and SLE partly through modulation of T cell and B cell numbers and functions (58, 68, 162, 202). Although changes in immune function have been observed in the context of calorie restriction, it is not clear whether these changes confer protection against infectious agents. In a study of salmonella infection in mice, calorie restriction—but not protein energy malnutrition—reduced mortality in infected mice (159). Calorie restriction also expedited recovery from *Streptococcus zooepidemicus* lung infection in mice (52), but increased mortality in a mouse model of polymicrobial peritonitis (203).

Comparison of calorie restriction immune effects to those in protein energy malnutrition and anorexia highlights the importance of specific macro- and micronutrients to the development and maintenance of the immune system. This contrast is especially apparent when comparing the effects of calorie restriction to protein energy malnutrition. In calorie restriction experiments, the main intervention is to lower the overall caloric consumption while maintaining equivalent vitamin and mineral content. In protein energy malnutrition studies, the experimental diet is specifically designed to contain fewer calories from protein but to contain the same amount of vitamins and minerals as the control diet. However, in studies of humans suffering from PEM, it was difficult to extricate the effects of protein deficiency from that of micronutrient deficiency. Although the majority of studies of malnutrition have focused on protein-calorie malnutrition, there is an increasing body of work which links immune function to specific micronutrients such as vitamin A. A series of studies conducted in 1980s and 1990s, which showed that Vitamin A supplementation reduced mortality rates and hastened recovery in South

African children with measles (86) and reduced overall mortality in malnourished children (198). Although the mechanism behind this protective effect is not clear, it has been shown that vitamin A deficiency can disrupt the development and function of the innate and adaptive immune systems (137). Mice fed a Vitamin-A-deficient diet have a significantly reduced ability to produce protective T_H2-related IgG1 antibodies in response to helminth infection (27). The deficiency in IgG1 production in Vitamin A-deficient mice is partly due to shift from T_H2 cytokine secretion (i.e. IL-4) to T_H1 cytokine secretion in the helper T cell compartment (28, 29), a reduction of T helper cell numbers and a reduction in clonal expansion of B cells in response to antigen (197). Vitamin A primarily exerts its effects through its retinoic acid (RA) metabolite and the retinoic acid receptor (RAR) nuclear hormone receptor. RA has been shown to facilitate the development of adaptive T regulatory cells in and inhibit the differentiation of T_H17 cells within mucosal associated lymphoid tissue (12, 43, 48, 144, 201, 236). A more recent study by Hall et al. revealed a more nuanced role for RA by showing that it was also necessary for the generation of the pro-inflammatory T_H17 cell response to *Toxoplasma gondii* infection and OVA immunization in a RAR-dependent manner (79). These findings contrast with previous studies which showed that it ameliorated mouse models of autoimmune disease through enhancement of the T regulatory cell suppressive response and regulation of the T_H17 response. This indicates that Vitamin A can either enhance or suppress inflammatory cell function depending on disease context. Other vitamins have also been associated with the development of autoimmune disease. For instance, Vitamin D has already been implicated in the pathogenesis of SLE and serum levels of this vitamin are reduced in SLE patient populations and negatively correlate with disease severity (177).

Immunity and Nutrition: Immunity in the Setting of Dietary Excess

The study of immune function in the context of dietary excess has been an area of intense study over the last two decades and the understanding of immunity in this setting is much more developed than in the setting of malnourishment. As described above, the adipose tissue has been a primary focus of studies in this field. Once thought to be an inert lipid storage depot, adipocytes are now known to influence the activity of many other organ systems through secretion of chemokines, cytokines and hormones. One of these affected systems—the immune system—has been an area of intense research within the diabetes field over the last decade. Although the relationship between inflammation and diabetes has been known since the late 1800s, the modern era of investigation in this area began in 1993 when Spiegelman et al. showed that the pro-inflammatory cytokine TNF- α contributed to insulin resistance in the adipose tissue of obese rodents and humans (82). The innate immune system was first implicated in obesity in 2003 by Ferrante *et al.*, who showed that adipose tissue macrophage accumulation was associated with TNF- α expression and insulin resistance (223). Since 2003, several other leukocyte classes and subtypes have been shown to reside in adipose tissue and influence obesity and diabetes. T regulatory cells (62), M2 macrophages (153) and eosinophils (234) have been shown to be protective, whereas neutrophils (205), mast cells (74), M1 macrophages(153), B cells (224), T_H17 cells (246), and CD8⁺ cells (151) exacerbate obesity and insulin resistance.

The rise in obesity rates in the United States have been accompanied by a rise in the prevalence in autoimmune disease, and several studies have offered clues regarding potential ties between these trends. In one study, HFD-feeding exacerbated mouse models of multiple sclerosis and colitis in an T_H17 cell dependent manner (226), but the mechanistic link between obesity and T_H17 cell activity has not been elucidated. One potential link is the satiety-signaling

hormone leptin. This hormone is produced by adipocytes and serum levels increase as body fat increases. Leptin's primary role is to signal satiety through receptors in the hypothalamic feeding center, but recent studies have shown effects in the periphery, including in the immune system. T cells express leptin receptor and T_H17 cell differentiation is enhanced by addition of leptin to Naïve CD4⁺ T cell cultures (47). Furthermore, leptin injections exacerbate disease in a mouse model of arthritis (47). Leptin deficiency and neutralization protects against allograft rejection (138) and EAE (69, 182) through a reduction of the T_H1 and T_H17 cellular response. Additionally, leptin acts as a negative regulator of T regulatory cell development and function (163). In humans, increased leptin levels have been observed in patients with psoriasis (220), rheumatoid arthritis (156) and lupus (70).

The evolutionary advantage for increased suppressive T regulatory cell activity in the fasting (low leptin) state is not immediately apparent. Since the generation of an immune response necessitates significant energy expenditure, it is possible that T regulatory cells contribute to energy conservation by immune system activity when food intake is low. Conversely, leptin may act as a signal to pro-inflammatory T_H1 and T_H17 cells that energy resources are sufficient to mount an immune response. Mice lacking leptin or leptin receptor have impaired immune function and fasted wild-type mice have decreased delayed-type hypersensitivity response to antigens (69). Addition of exogenous leptin is sufficient to restore immune function in fasted mice or leptin-deficient mice, which suggests that immune cells are directly affected by the lack of leptin and may not directly sense a lack of nutrients.

Metabolic Syndrome, Insulin Resistance and Obesity in Lupus

There have been several case reports describing insulin resistance in humans suffering from SLE. Many of the earliest studies referenced an insulin resistance type b phenomenon in

which autoantibodies against the insulin receptor or free insulin impaired normal insulin signaling (63, 93). One of the earliest cases of lupus-related type B insulin resistance was reported in 1981, in which a 22 year-old female who had recently been diagnosed with SLE was found to have hyperinsulinemia and diabetes that responded to corticosteroid treatment (221). Many other cases of lupus-related type B insulin resistance have been published in the literature (11, 15, 72, 97, 109, 136, 155, 158, 170). Collectively, these case studies described patients that presented with hyperinsulinemia accompanied by deranged glucose homeostasis. In two thirds of patients, insulin receptor antibodies can block insulin binding, resulting in hyperglycemia (25). In the remaining third, the insulin receptor antibodies can potentiate the insulin response, causing episodes of hypoglycemia. Patients with type B insulin resistance can vacillate between hyperglycemia and hypoglycemia during the course of the disease, but blood glucose can be effectively managed using conventional SLE therapies such as corticosteroids and cyclosporine.

Classical insulin resistance (insulin resistance that is secondary to faulty signaling downstream of the insulin receptor) is also increased in incidence in the SLE population. Several studies have shown that SLE patients have increased fasting insulin compared to healthy controls (3, 38, 116, 167, 188, 209, 210). This hyperinsulinemia is accompanied by increased levels of leptin and a paradoxical increase in adiponectin (176). The instigating factor for hyperinsulinemia in the context of SLE is not clear. One candidate—adiposity—could be a contributing factor. Although early studies showed higher body mass index (BMI) in cohorts of lupus patients (100, 101), subsequent studies failed to show significant differences in BMI between SLE patients and healthy controls (116, 167, 209, 210). Another possible contributing factor could be the use of corticosteroids for the management of SLE. Corticosteroids have been shown to cause changes in adipose distribution, hyperglycemia, beta cell impairment and

inhibition of insulin receptor signaling (94), so it is logical to conclude that they may cause the metabolic dysfunction seen in SLE patients. However, studies of this subject have failed to yield a correlation between cumulative corticosteroid use and metabolic disease in SLE patient cohorts (37, 38, 116, 210). Finally, the systemic inflammation present in the setting of SLE could be a contributing factor. Studies in this area have been more fruitful and have yielded clues regarding the immunological changes that contribute to insulin resistance in SLE. One study showed a strong positive correlation between the serum levels of the pro-inflammatory cytokine IL-18 and insulin levels in a lupus patient cohort (209), but another study failed to show a correlation between serum levels of IL-6 or TNF- α and insulin resistance. Serum levels of IL-18, a proinflammatory cytokine that is produced by macrophages, dendritic cells and Kupffer cells, has been shown to correlate with insulin resistance (208). IL-18 is important in the activation of NK cells and T cells and serum IL-18 levels have been shown to be associated with many components of the metabolic syndrome in the general obese population (85) and in the SLE patient population (208).

Only one study of insulin resistance in a mouse model of lupus has been published (185). This study by Ryan et al. showed that lupus-prone NZB/W F1 mice had hyperinsulinemia, hyperleptinemia and higher glucose intolerance than control parental strain mice. In the following chapter, we present data regarding glucose intolerance in the B6.SLE.1.2.3 mouse model of lupus which develop adipose tissue insulin resistance and glucose intolerance when fed low-fat diet.

CHAPTER II

AUTOIMMUNE-MEDIATED INSULIN RESISTANCE IN A MOUSE MODEL OF SYSTEMIC LUPUS ERYTHEMATOSUS

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease in which pathogenic autoantibodies disrupt the functions of various organs and organ systems. Causes of SLE mortality can be clustered into two main groups: early mortality and late mortality (24, 61, 214). Early causes of mortality include glomerulonephritis, infection and neurological disease, whereas cardiovascular disease (CVD) is the main cause of late mortality (95, 181). Clinical management of SLE has improved over the last decades, shifting the mortality burden from early causes to late causes. As this burden shifts, it is important to understand the factors that contribute to the development of CVD in SLE patients. SLE patients have been shown to have a higher prevalence of traditional CVD risk factors such as hyperlipidemia or hypertension and non-traditional, SLE-specific risk factors such as autoreactive immunoglobulin or corticosteroid therapy (24). Additionally, metabolic syndrome is more prevalent in the SLE population (37, 56, 116, 186) and may contribute to the increased CVD burden in these patients.

The metabolic syndrome is characterized by the presence of obesity, IR, hypertension, and dyslipidemia (54, 96). IR, a central component of type 2 diabetes mellitus (T2DM), is thought to be caused by chronic inflammatory processes involving complex interactions between cells of the metabolic and immune systems. Several studies have yielded crucial information implicating visceral adipose tissue as a main focus of the chronic inflammation that can contribute to IR and T2DM. Pro-inflammatory M1 macrophages accumulate in the adipose tissue of obese mice (223), and blocking macrophage accumulation attenuates IR in these

animals (222). Subsequent studies have shown that the adaptive immune system mediates insulin resistance in obesity. CD8⁺ T cells and B cells exacerbate insulin resistance in obese mice, whereas regulatory T cells protect against the development of insulin resistance (62, 151, 224, 225).

In the only other study of metabolic syndrome in an SLE model of which we are aware, Ryan *et al.* (185) showed that the (NZB/W)F1 lupus-prone strain has increased adiposity, increased adipose tissue macrophage infiltration and worsened glucose intolerance. However, the contributions of the adaptive immune system to this metabolic phenotype was not examined and merits further study.

We examined the pathogenesis of metabolic syndrome in the B6.SLE mouse model of SLE. The B6.SLE congenic strain is derived from the NZM2410 [(NZB/W)F1 X NZW] model of SLE, which develops severe lupus and nephritis in both genders (139). Through linkage analyses, Morel *et al.*, identified three major genomic intervals linked to lupus susceptibility in the strain (139). Using these three chromosomal intervals, termed *Sle1*, *Sle2* and *Sle3*, the investigators made triple congenic mice on the C57Bl/6 background (140, 141). The B6.*Sle1.2.3* strain, also known as the B6.SLE strain, displays a fully penetrant lupus phenotype similar to that of humans. In the current study, we present evidence that accelerated insulin resistance and disrupted glucose homeostasis in B6.SLE may be mediated by pathogenic B cells.

Materials and Methods

Mice. B6.*Sle.1.2.3* mice, hereafter referred to as B6.SLE mice, were a generous gift of Edward Wakeland (UTSW, Dallas, TX) and have been previously described (139). C57BL/6 mice (B6) were originally purchased from Jackson Labs (Bar Harbor, ME), and were bred in the animal facility at Vanderbilt University Medical Center. All procedures involving animal subjects received prior approval from the Vanderbilt University Institutional Animal Care and Use Committee.

Diet. B6 and B6.SLE mice were co-housed after weaning and fed low fat diet (LFD, 10% Kcal from fat, D12450B, Research Diets, New Brunswick, NJ) or HFD (HFD, 45% Kcal from fat, D12451, Research Diets, New Brunswick, NJ) *ad libitum*.

Glucose Tolerance Tests. Study mice were fasted for 5-6 hours on paper bedding before their tails were nicked under isoflurane anesthesia. Mice were rested for 20 minutes and then injected i.p. with 1.25 g/kg lean body mass glucose. Blood glucose was measured at baseline and at 15, 30, 45, 60, 90 and 120 minutes after injection using a One Touch Ultra glucometer.

Anti-dsDNA Antibody ELISA. Serum titers of anti-dsDNA antibodies were measured using the protocol described by Shivakumar et al.(194). Nunc MaxiSorp plates were blocked with mBSA in 1X PBS (0.1 mg/ml) at 37° C for 30 minutes. The plate was then washed with PBS and coated with 50 µg/mL dsDNA in PBS at 37° C for 30 minutes. The plate was washed twice with PBS and blocked overnight at 4° C with blocking buffer (3% BSA, 3 mM EDTA, and 0.1% gelatin in PBS). The plates were washed twice with PBS. The serum was diluted 1:1000 in serum diluent (2% BSA, 3mM EDTA, 0.05% Tween 20 in PBS) and added to plate and incubated 2 hours at RT on an orbital shaker. The plate was washed twice in PBS-tween and twice in PBS. IgG-HRP (Promega, Madison, WI) was diluted 1:5000 in secondary diluent (1% BSA and 0.05% Tween in

PBS), added to the plate and incubated overnight at 4° C on an orbital shaker. The plate was washed twice with PBS-tween and twice with PBS and OptEIA TMB Substrate (BD Biosciences, San Diego, CA) was added to the plate and allowed to incubate. The reaction was quenched with 1 M phosphoric acid and the plate was promptly read at 450nm.

Islet Perifusion Assay. Pancreatic islets were isolated and assessed for secretory capacity as previously described (22, 219, 227). Briefly, the pancreas was digested in a solution of collagenase P in Hank's Balanced Saline Solution (HBSS) using a wrist-action shaker. Digestion was quenched with ice-cold 10% FCS/HBSS. The digest was washed and islets were hand-picked under microscopic guidance. Islet insulin secretion was analyzed in a dynamic cell perfusion system. The perfusion medium was Dulbecco's Modified Eagle Medium supplemented (Gibco) with 10 mM HEPES, 26 mM NaHCO₃ and 0.1% BSA. Fifty islet equivalents were placed in a chamber and washed under baseline media for 30 minutes prior to the experiment. Islets were then perfused for 9 minutes with 5.6 mM glucose (Sigma, D16), followed by 30 minutes with 16.7 mM glucose, 21 minutes with 5.6 mM glucose, 9 minutes with 16.7 mM glucose + 50 µM isobutylmethylxanthine (IBMX, Sigma, I5879-1G), 21 minutes with 5.6 mM glucose, 9 minutes with 20 mM KCl and 21 minutes with 5.6 mM glucose. The effluent fractions were collected at 3-minute intervals using an automatic fraction collector. The insulin concentration of each fraction was measured by radioimmunoassay (RI-13K, Millipore).

Immunoglobulin ELISA. Serum IgG was measured using conventional sandwich ELISA. Briefly, MaxiSorp ELISA plates (Nalge Nunc, Rochester, NY) were coated overnight with 0.5 µg/mL IgG heavy and light chain (Southern Biotech, Birmingham, AL). Plates were blocked 1h with 10% FBS in 1X PBS and serum samples or mouse reference serum (Bethyl Laboratories, Montgomery, TX) were incubated overnight at 4° Celsius. Detection antibody [0.5 µg/ml biotin-

conjugated anti-IgM , -IgG1, -IgG2c (Southern Biotech, Birmingham AL), or anti-IgG HRP-conjugated antibody (1:2500) (Promega, Madison, WI)] was incubated 2 hours at RT. Streptavidin-HRP (1:2500)(Sigma, St. Louis, MO) was incubated in IgM, IgG1, or IgG2c wells for 1 hour at RT before plates were developed with OptEIA TMB Substrate (BD Biosciences, San Diego, CA).

BAFF and Insulin ELISA. Serum BAFF was measured using a Quantikine Mouse BAFF/BlyS/TNFSF13B ELISA Kit (R&D Systems, Minneapolis, Minnesota, USA). Serum insulin was measured using a Rat/Mouse Insulin ELISA kit (Millipore, St. Charles, Missouri, USA).

Anti-insulin antibody ELISA. Anti-insulin antibody titers were measured as described in *Rojas et al.* (180). MaxiSorp ELISA Plates (Nalge Nunc, Rochester, NY, USA) were coated overnight at 37° C with human insulin at a concentration of 1 µg/mL in borate buffered saline (100mM Boric Acid, 25mM Sodium Borate, 150mM NaCl, pH 8.2-8.5). Plates were washed 3 times with PBS-Tween, and serum was diluted 1:100 in PBS-Tween. In order to measure the presence of polyreactive autoantibodies, sample serum was concurrently diluted 1:100 in PBS-Tween spiked with 50 µg/mL human insulin. Both spiked and unspiked dilutions of sample serum were incubated 1 hour at room temperature on an orbital shaker. Plates were washed 4 times in PBS-Tween and an alkaline phosphatase conjugated anti-IgG antibody (Southern Biotech, Birmingham AL, USA) was added to the plate at a final dilution of 1:500 in VBS containing FBS (140 mM NaCl, 5 mM sodium barbital, 15 mM sodium azide, and 0.5% FBS). The plate was washed 4 times with PBS-tween and PNPP substrate (Sigma-Aldrich, St. Louis, MO, USA) was added and the plate was read at 405 nM when sufficient development had occurred.

Tissue Preparation. Perigonadal fat pads were minced and digested for 30 minutes at 37° C with agitation in 1 mg/ml collagenase in HBSS (Sigma, St. Louis, Missouri). Digested tissue was passed through a 40 micron filter and leukocytes were isolated from the interface of a 40%/60% Percoll gradient (GE Healthcare, Piscataway, NJ). Livers were collected and crushed through a 40 micron filter and leukocytes were isolated from the interface of a 40%/60% Percoll gradient.

Flow Cytometry. Single-cell suspensions of liver mononuclear cells and stromal vascular cells were isolated as described above. In order to minimize non-specific binding of flow antibodies, cells were blocked for 15 minutes at RT with a 1:200 dilution of Fc receptor block (BD Biosciences, San Diego, CA) in a FACS buffer consisting of 1X HBSS, 1% BSA, 4.1663 mM sodium bicarbonate and 3.0765 mM sodium azide. The following fluorescently-labeled antibodies were diluted 1:200 in FACS buffer and incubated with the cells for 45 minutes at 4° C: CD19-PECy7, B220-A450, TCR β -PECy7, IgG-FITC, IgM-PE, IgD-APC, CD80-APC, and F4/80-PECy7, CD8-FITC, CD4-APCCy7, and BAFFR-PE (BD Biosciences, San Diego, CA). The labeled cells were washed 3 times with FACS buffer and fixed overnight at 4° C in a solution of 2% paraformaldehyde in PBS. Labeled cells were analyzed on a MACSquant seven-color flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) and data was analyzed with FCS Express 4 (De Novo Software, Los Angeles, CA).

Immunoblotting. Mice were fasted on paper bedding for four hours and then injected with 1U/Kg body weight insulin. Fifteen minutes later, mice were sacrificed and liver, soleus, gastrocnemius and perigonadal fat pads were removed and freeze clamped in liquid nitrogen. Fat was homogenized in a solution of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, 5 mg/mL protease inhibitor, 50 mM NaF, and 5 mM sodium pyrophosphate and centrifuged for 10 minutes at 13,000 RPM at 4° C. The

lysate was run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was probed with Akt primary rabbit antibody (1:1000)(Cell Signaling, Danvers, MA) or pAKT primary rabbit antibody (1:1000)(Cell Signaling, Danvers, MA) and incubated overnight at 4° C. The membrane was then incubated for 1 hour at RT with anti-beta-actin antibody (1:1000)(Sigma Aldrich, St. Louis, MO) followed by incubation with goat anti-rabbit 800LT conjugated secondary antibody (1:5000)(Li-Cor, Lincoln, NE) and goat anti-mouse 680LT conjugated secondary antibody (1:20,000) and for 1h at RT. Gels were scanned using a Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE) and bands were quantified using Odyssey Version 3.0 software (Li-Cor, Lincoln, NE).

Serum cholesterol, serum triglycerides, and liver triglycerides. Mice were fasted on paper bedding for 4 hours and blood was collected via the retro-orbital sinus under isoflurane anesthesia. Blood was allowed to clot for 20 minutes and was subsequently spun at 13,000 RPM for 20 minutes at 4° C. Serum was collected, protease inhibitor cocktail was added and the samples were stored at -80° C. Serum was thawed and diluted 1:100 with dH₂O and triglyceride and cholesterol were measured using a Cholesterol and Triglyceride Enzymatic Assay according to the manufacturer's protocol (Cliniqa Corporation, San Marcos, CA).

Lipids were extracted from the liver using the method of Folch-Lees (64). Individual lipid classes were separated by thin layer chromatography and visualized by rhodamine 6G. Triglycerides were scraped from the plates and methylated using BF₃ /methanol as described by Morrison and Smith (142). The methylated fatty acids were extracted and analyzed by gas chromatography. Gas chromatographic analyses were carried out on an Agilent 7890A gas chromatograph equipped with flame ionization detectors, a capillary column (Supelco,

Bellefonte, PA). Helium was used as a carrier gas and fatty acids were identified by comparing the retention times to those of known standards.

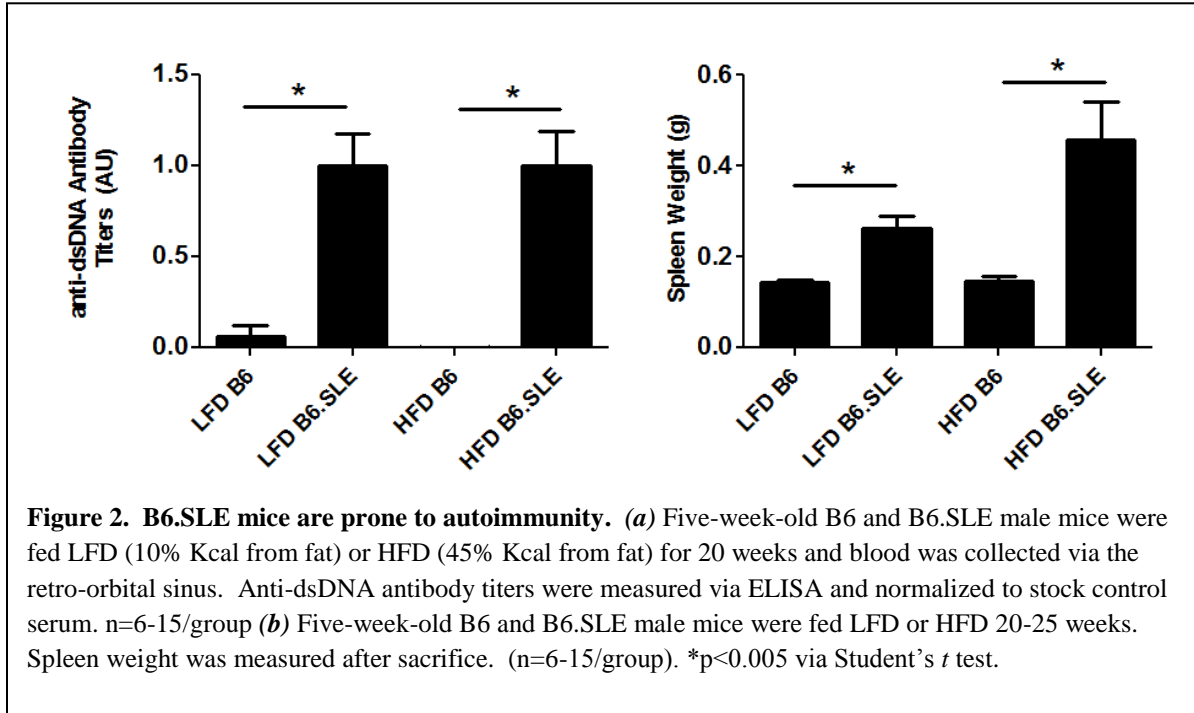
Adipocyte size measurement. Peri-gonadal fat pads were collected and fixed in a phosphate-buffered solution containing 10% formalin. Fat was embedded in paraffin, sectioned and stained with hematoxylin and eosin. Adipocyte size was quantified by capturing 4 independent fields per blinded slide and measuring adipocyte area using ImageJ software (National Institutes of Health, Bethesda, MD). Average adipocyte size was calculated and expressed as $\mu\text{m}^2/\text{adipocyte}$.

Multiplex adipokine assay and C-Peptide ELISA Study mice were fasted for 4 hours on paper bedding and blood was collected from the retro-orbital sinus under isoflurane anesthesia. Blood was allowed to clot for 20 minutes and was subsequently spun at 13,000 RPM for 20 minutes at 4° C. Serum was collected, protease inhibitor cocktail was added and the samples were stored at -80° C. A Milliplex mouse adipokine assay (Millipore, Billerica, MA) was used to measure insulin, leptin, resistin, IL-6 and TNF-alpha according to manufacturer instructions. Serum C-Peptide was measured using Millipore C-Peptide 2 ELISA kit according to manufacturer's instructions.

Statistics. Statistical significance for GTT data was calculated with a two-way ANOVA for repeated measures. All other significance was calculated with a Student's *t* test comparing mice within diet groups. All calculations were completed with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Unless otherwise noted, all data was expressed as mean +/- SEM.

Results

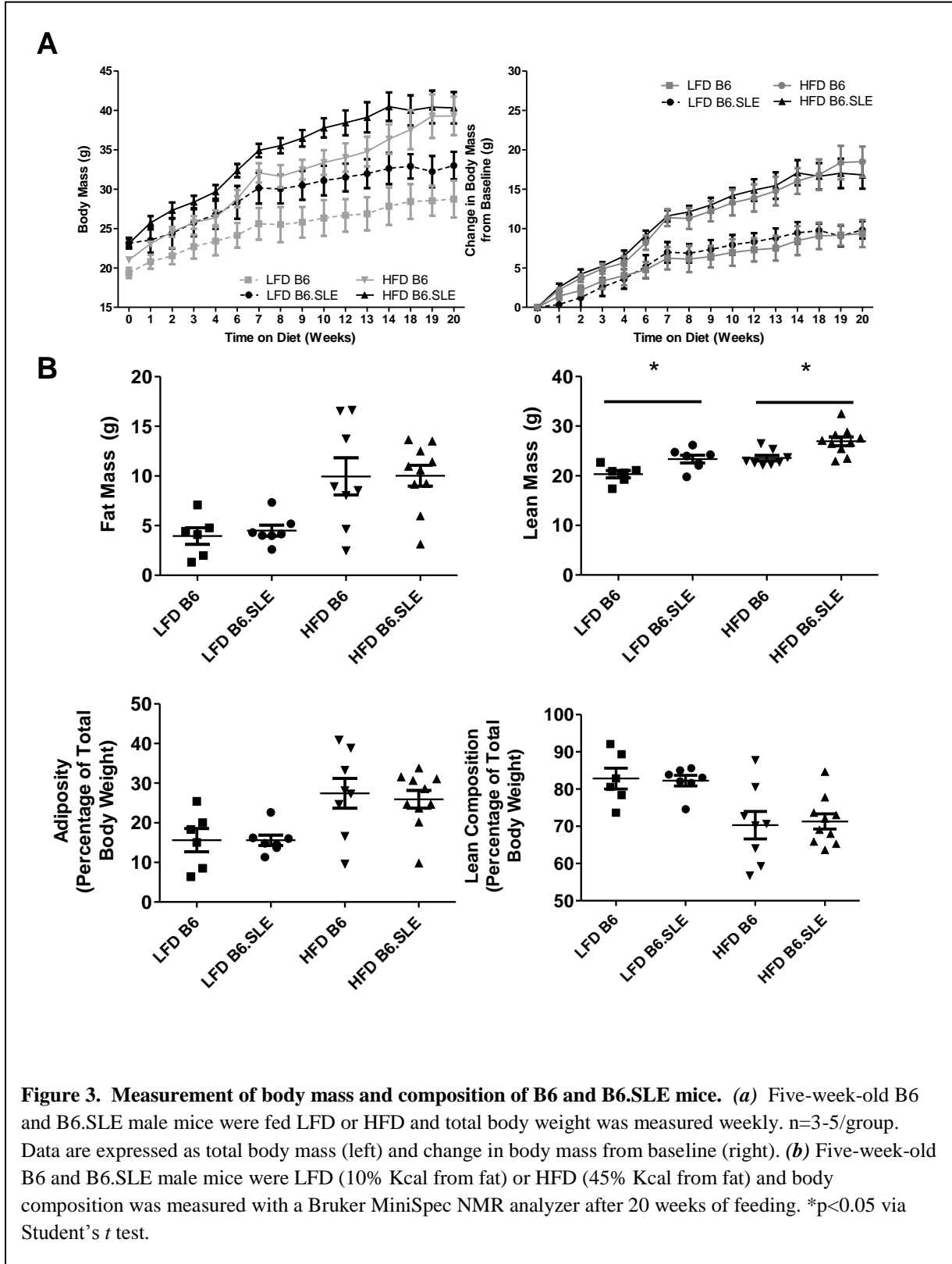
B6.SLE Mice Are Prone to Autoimmunity. Increased production of pathogenic anti-dsDNA antibodies and splenomegaly are hallmarks of autoimmune disease in B6.SLE mice. Five-week-old male B6 and B6.SLE mice were placed on LFD or HFD. After twenty weeks of diet feeding,



both anti-dsDNA antibody titers and spleen weight (Figure 2) were significantly increased in B6.SLE mice relative to their respective controls. Interestingly, splenomegaly was more pronounced in HFD-fed B6.SLE mice than LFD-fed B6.SLE mice but there was no difference in anti-dsDNA antibody titers between B6.SLE mice of each diet group. Although the markers of autoimmunity were significantly increased, there was no increased mortality in B6.SLE mice after 20 weeks of feeding as compared to B6 controls (data not shown).

Weight and Body Composition of B6.SLE Mice Varies from B6 Controls. Total body mass was measured weekly after LFD and HFD was initiated (Figure 3a, left panel). The baseline weights

of B6.SLE mice in both diet groups were higher than their respective controls and remained



elevated for the duration of the experiment. Weight gain, as expressed as change in body weight from baseline (Figure 3a, right panel), was not significantly different between B6 and B6.SLE mice in each diet group. Lean body mass and adipose tissue mass were assessed in all groups after 20 weeks of feeding (Figure 3b). Adiposity was not significantly different between LFD-fed B6.SLE and B6 mice at this time point. However, lean body mass was significantly higher in LFD- and HFD-fed B6.SLE groups as compared to B6 controls and partially accounted for the increased body weight observed in LFD-fed B6.SLE mice in Figure 3a.

Glucose Homeostasis and Insulin Receptor Signaling is Altered in B6.SLE Mice. Glucose tolerance tests were conducted in male B6.SLE and B6 mice after 20 weeks of LFD or HFD feeding (Figure 4a and b). Glucose intolerance was significantly higher in B6.SLE mice than their respective B6 controls in the LFD-fed groups when data was expressed as absolute blood glucose level (Figure 4a) or as the area under the curve (Figure 4b). Interestingly, the glucose curve of LFD-fed B6.SLE mice approached the curve of HFD-fed B6 mice.

In order to determine whether insulin signaling is disrupted in metabolically active tissues in B6.SLE mice, Western blotting was used to measure the phosphorylated-Akt-to-Akt ratio in the perigonadal fat pads, liver, soleus muscle and gastrocnemius muscle. Akt is a critical component of the insulin signaling pathway and becomes phosphorylated after the insulin receptor is ligated, ultimately causing translocation of the GLUT4 glucose transporter to the cell surface. The pAkt-to-total-Akt ratio was significantly reduced in the adipose tissue of LFD-fed B6.SLE mice relative to B6 controls (Figure 4c), indicating that insulin signaling is impaired in the adipose tissue of LFD-fed SLE mice. No significant difference in Akt phosphorylation was seen in LFD-fed strains in the liver, gastrocnemius or soleus muscle although trends toward lower ratios of phosphorylation

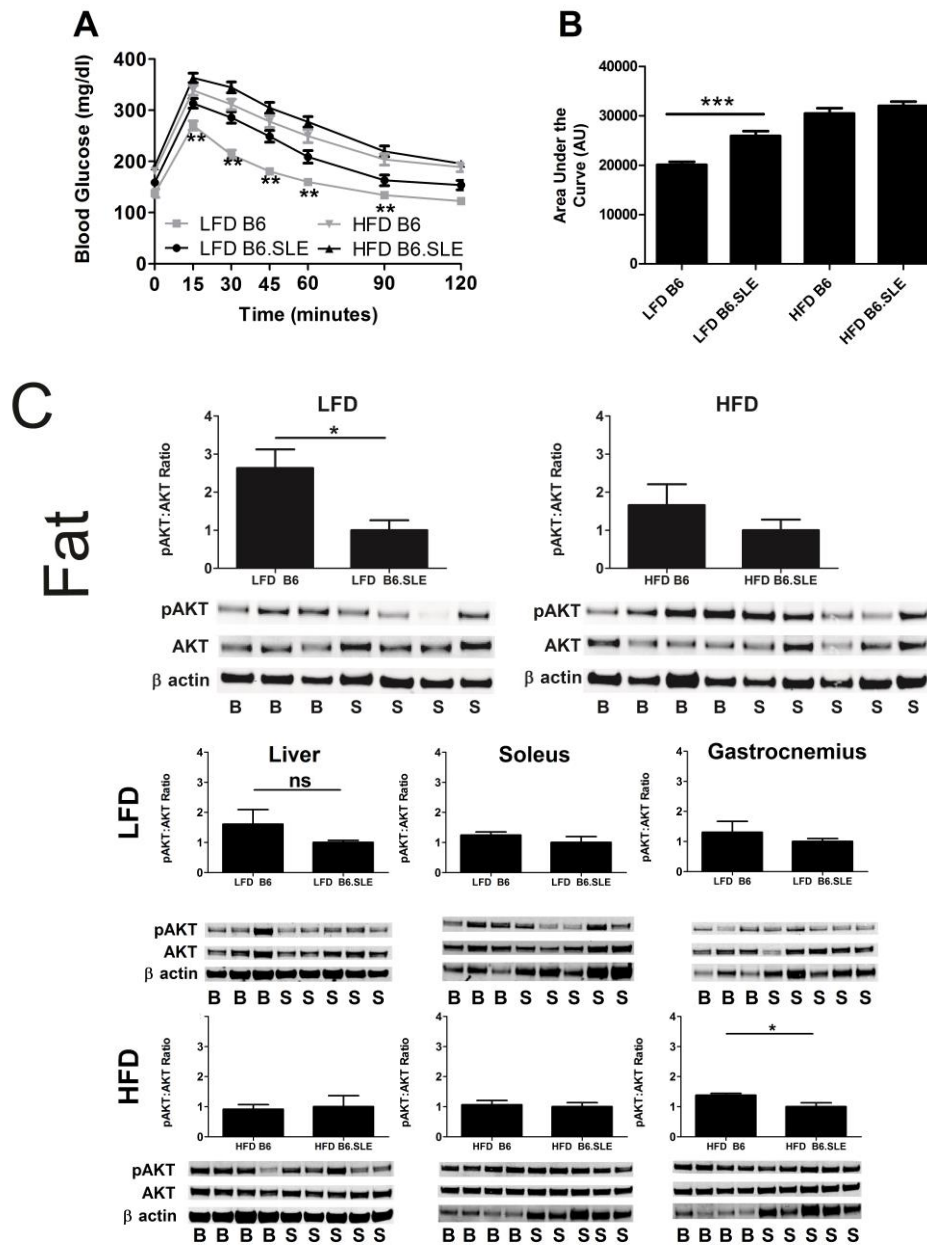
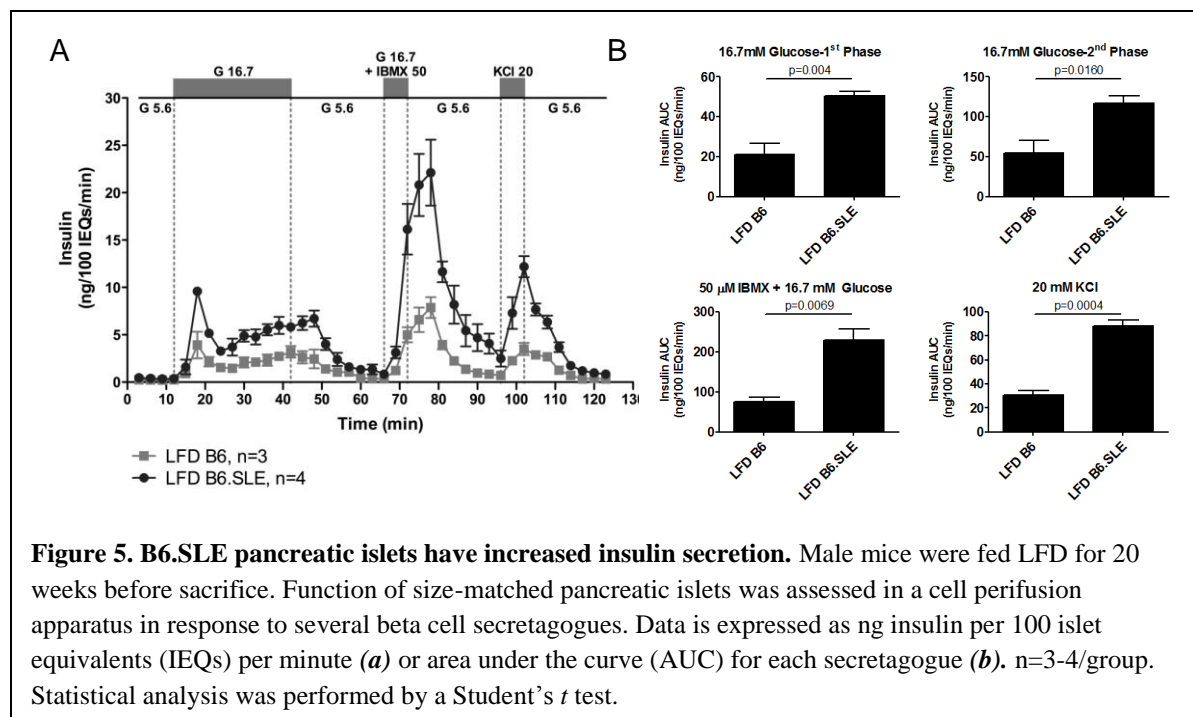


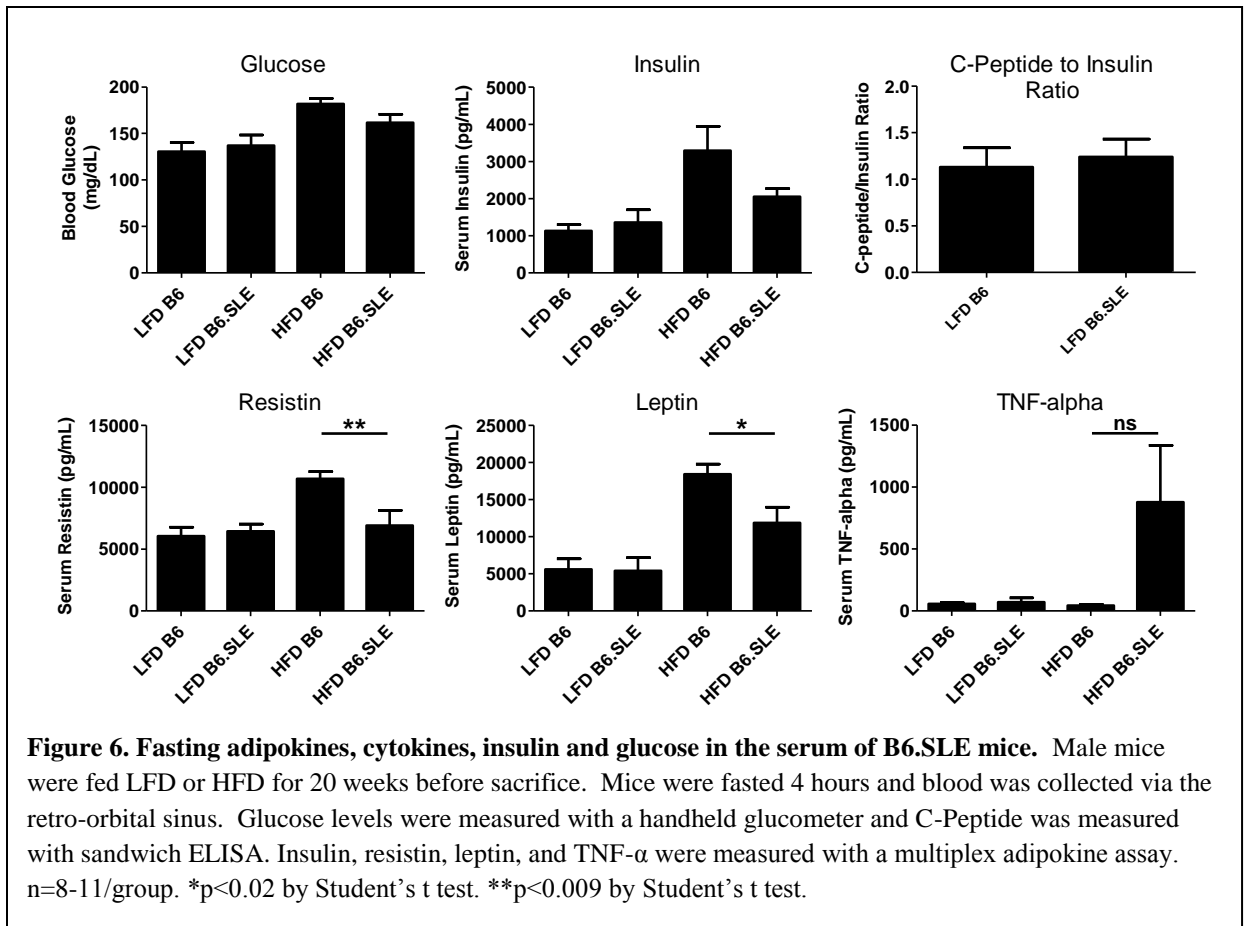
Figure 4. Glucose homeostasis and insulin receptor signaling is impaired in B6.SLE mice. Five-week-old B6 and B6.SLE male mice were fed low fat diet or high fat diet for 20 weeks. After 5-6 hours of fasting, mice were injected with 1.25 g glucose per kg lean body weight and blood glucose was measured using a One Touch Ultra glucometer. $n=16-25$ mice/group in four independent experiments. Data are expressed as blood glucose over time (a) or area under the glucose excursion curve (b). $**p<0.0001$ between LFD-fed B6.SLE and B6 mice via two-way ANOVA. $***p<0.0001$ via Student's *t* test. (c) After 20 weeks of HFD or LFD feeding, mice were fasted for four hours and then injected with 1U/Kg lean body weight insulin. Fifteen minutes later, mice were sacrificed and liver, gastrocnemius, soleus and perigonadal fat was removed and freeze clamped in liquid nitrogen. Tissue lysate was analyzed by Western blot with Akt and pAkt antibodies. Data is expressed as integrated intensity of Akt or the ratios of the integrated intensity of pAkt and Akt. $n=3-5$ mice/group. Values are expressed as mean \pm SEM. $*p<0.04$ via Student *t* test.



in B6.SLE mice were observed. No significant differences in pAkt ratios were seen between strains in any of the tissues in HFD-fed groups.

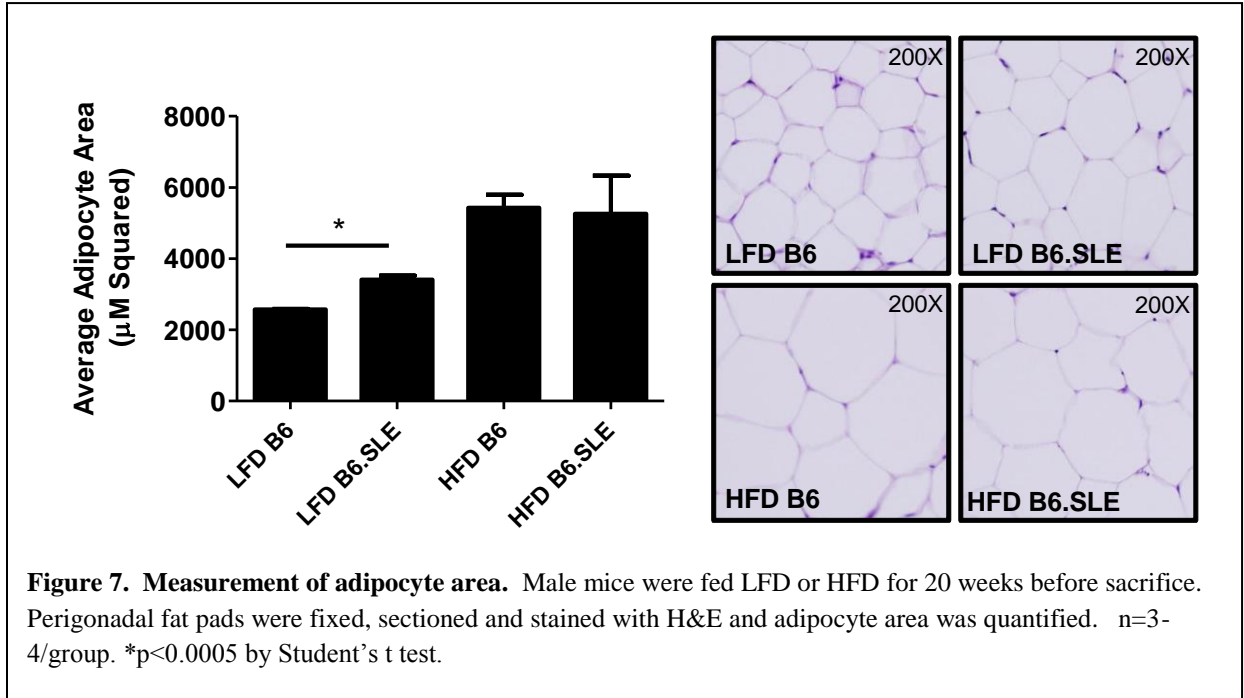
B6.SLE Islets Have Increased Insulin Secretion. In order to determine whether the observed glucose intolerance is due to impaired beta cell function, pancreatic islets were isolated from LFD-fed B6 and B6.SLE mice and responses to several beta cell secretagogues were measured in an islet perfusion system. Figure 5a shows that insulin secretion was significantly increased in the islets of LFD-fed B6.SLE mice in response to 16.7 mM glucose, 16.7 mM glucose + 50 μ M IBMX, and 20 mM KCl, ruling out beta cell defects in glucose sensing, metabolism or granule exocytosis in LFD-fed B6.SLE mice.

Circulating Adipokines and Cytokines are Not Different in LFD-fed B6.SLE Mice. To determine if circulating adipokines and cytokines contribute to glucose intolerance in B6.SLE mice, fasting levels of leptin, resistin, IL-6, TNF α and insulin were measured using a cytokine



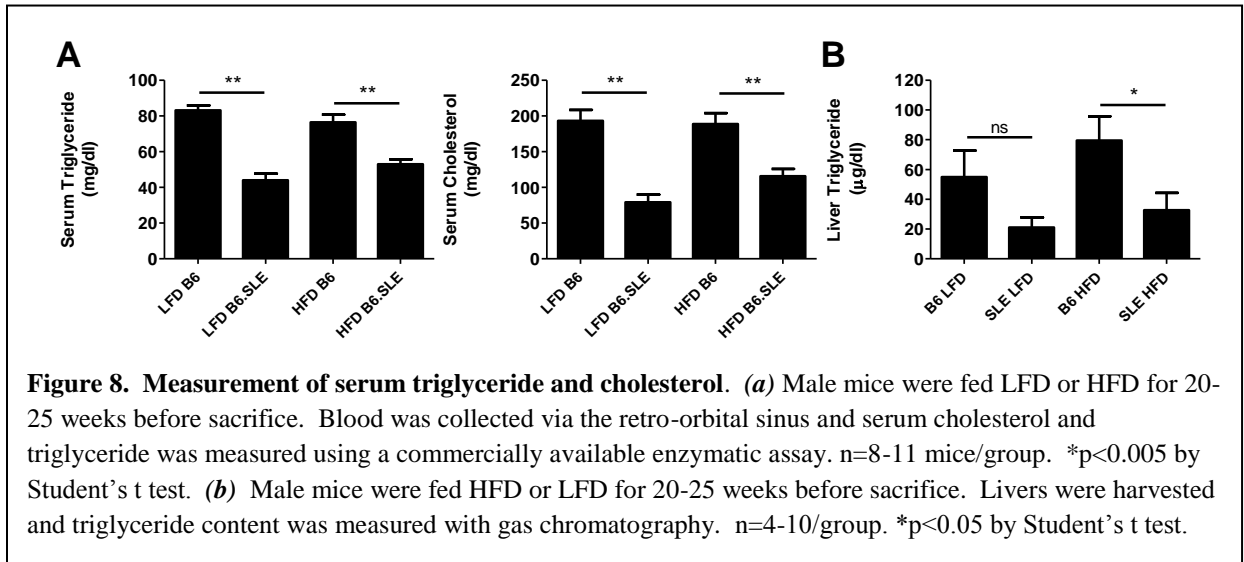
bead array, and fasting glucose was measured using a handheld glucometer. As seen in Figure 6, no differences in fasting insulin or fasting glucose were observed between strains in the LFD-fed groups and there was a non-significant trend toward higher glucose and insulin in HFD-fed B6 mice relative to HFD fed B6.SLE mice. Insulin clearance was estimated by measuring the ratio of fasting serum insulin to fasting serum C-peptide 2 in LFD-fed mice. C-peptide 2 is cleaved from the pro-insulin molecule during the formation of insulin (184). C-peptide 2 is cleared in the kidney (242) and insulin is primarily cleared in the liver (53), and the ratio of these two molecules can be used to estimate hepatic clearance of insulin. There was no difference in the C-peptide to insulin ratio between B6.SLE and B6 mice, which suggests that SLE disease does not affect the rate of insulin clearance in B6.SLE mice.

Levels of serum resistin and leptin in LFD-fed groups were indistinguishable, and levels of resistin and leptin were significantly lower in HFD-fed B6.SLE mice relative to HFD-fed B6 controls. There was a trend toward higher TNF- α in HFD-fed B6.SLE mice relative to controls, but no increase in serum TNF-alpha was seen in LFD-fed B6.SLE mice. IL-6 levels were below the level of assay detection in all groups. Collectively, these results suggest that the glucose intolerance and adipose tissue insulin resistance observed in LFD-fed mice is not instigated by adipokines or cytokines normally associated with insulin resistance and may result from SLE-specific factors.



Adipocyte Size is Increased in B6.SLE Mice. Increased adipocyte size has been correlated with glucose intolerance and adipose tissue insulin resistance. In order to determine whether B6.SLE mice have increased adipocyte size, we measured adipocyte size in paraffin-embedded adipose tissue. As seen in Figure 7, average adipocyte size was significantly greater in LFD-fed B6.SLE

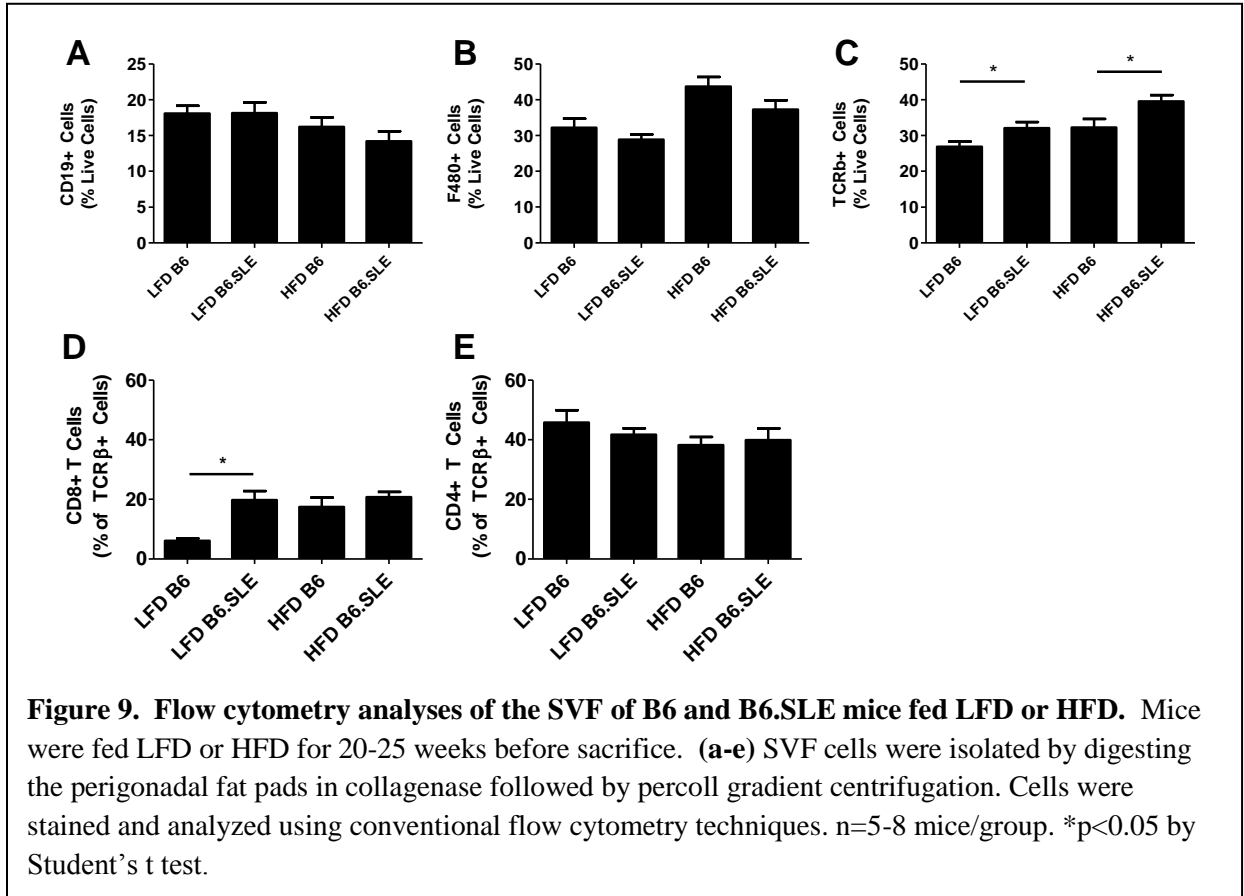
mice relative to B6 controls. There was no difference in average adipocyte size between HFD-fed B6.SLE mice and their controls.



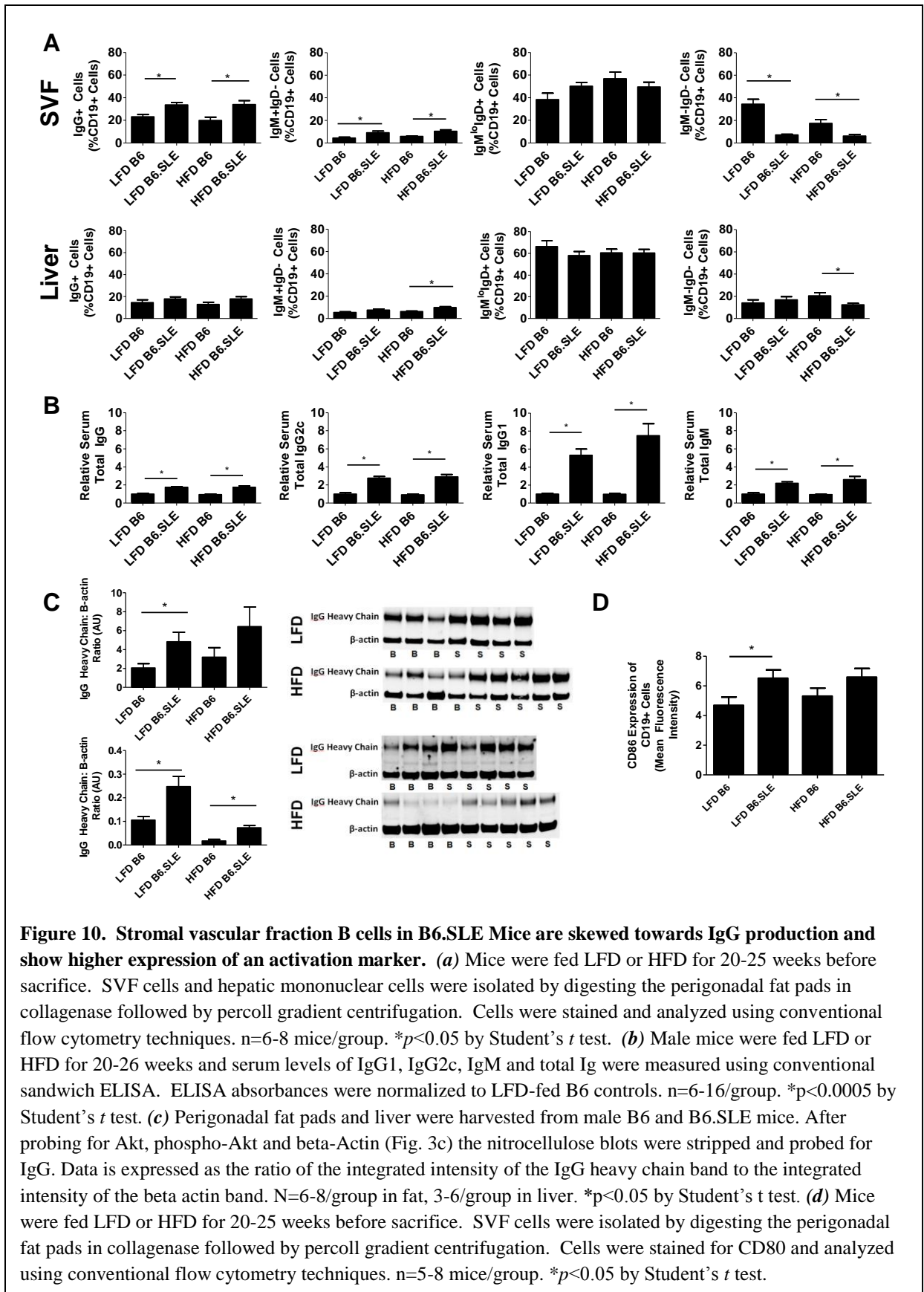
Serum Cholesterol, Serum Triglyceride, and Liver Triglyceride are Significantly Reduced in B6.SLE Mice. We measured serum cholesterol and triglycerides in study mice in order to determine whether lipid homeostasis is perturbed in B6.SLE mice. As seen in Figure 8a, serum cholesterol and triglyceride levels are significantly decreased in HFD- and LFD-fed B6.SLE mice relative to their respective controls. Liver triglyceride content (Figure 8b) is significantly decreased in HFD-fed B6.SLE mice relative to B6 controls and there is a non-significant trend towards reduced triglyceride content in the livers of LFD-fed B6.SLE mice.

Leukocyte Content in the Stromal Vascular Fraction Varies in SLE Mice. Cells of the adaptive and innate immune systems have been associated with adipose tissue dysfunction in mouse models of obesity. In order to examine the immune compartment in study mice, we used flow cytometry to assess the cell content of the SVF in LFD- and HFD-fed mice after 20 weeks of feeding. No significant difference was seen in the percentage of total CD19⁺ B cells in the SVF (Figure 9a) in either diet group. There were no differences in antigen presenting cell content of

the SVF between strains within each diet group as measured by F480, a marker of both macrophages and dendritic cells (Figure 9b). These findings indicate that F480+ cell infiltration—known to be associated with insulin resistance in obese mice—is not correlated with



glucose intolerance and adipose insulin resistance in B6.SLE mice. TCRβ⁺ T cell content in the SVF of HFD- and LFD-fed B6.SLE was significantly higher than their respective B6 controls (Figure 9c). Additionally, the proportion of CD8+ T cells—but not CD4+ T cells—in LFD-fed B6.SLE mice was significantly increased relative to controls and exceeded the percentages of CD8+ T cells in HFD-fed B6 mice (Figure 9d-e). This increase in CD8+ T cell parallels the results of Nishimura et al. in which HFD-feeding caused an increase in CD8+ T cells which correlated with insulin resistance in their study (151).



Stromal Vascular Fraction B Cells in B6.SLE Mice Are Skewed Towards IgG production.

Although no quantitative differences were observed in the CD19⁺ B cell population in study mice, we hypothesized that B6.SLE mice were predisposed to develop qualitative differences in the SVF-resident B cell population. Previous studies by Winer et al. ¹⁹ have shown that B cells contribute to adipose tissue insulin resistance through production of pathogenic IgG antibodies. We investigated whether SVF resident B cells had undergone class-switch recombination by using flow cytometry to quantify IgD, IgM and IgG expression on CD19⁺ B cells (Figure 10a). The percentage of class-switched IgG⁺ B cells in B6.SLE mice was significantly higher than B6 controls in each diet group. No differences in IgG⁺ B cell ratios were observed in the liver, indicating that these changes are specific to the gonadal fat pad. To determine whether systemic Ig levels were altered in B6.SLE mice, circulating total IgG, IgG1, IgG2c and IgM were measured in serum collected from male mice fed for 20-26 weeks (Figure 10b). LFD- and HFD-fed B6.SLE mice had significantly higher circulating total IgG, IgG1, IgG2c and IgM than their respective B6 controls. Perigonadal fat pad lysates were probed for IgG heavy chain in order to determine whether SLE mice had higher adipose tissue IgG deposition than B6 controls. As seen in Figure 10c, there was significantly more IgG heavy chain in the perigonadal fat pads of LFD-fed B6.SLE mice as compared to LFD-fed B6 controls. Furthermore, there was significantly more IgG deposition in the livers of LFD- and HFD-fed B6.SLE mice relative to their B6 controls. Finally, the expression of the activation marker CD80 was significantly increased in SVF B cells of LFD-fed B6.SLE mice (Figure 10d). Type B insulin resistance is caused by autoantibodies against insulin and the insulin receptor and has been associated with SLE (93). A modified ELISA was used to determine whether the glucose intolerant phenotype in B6.SLE

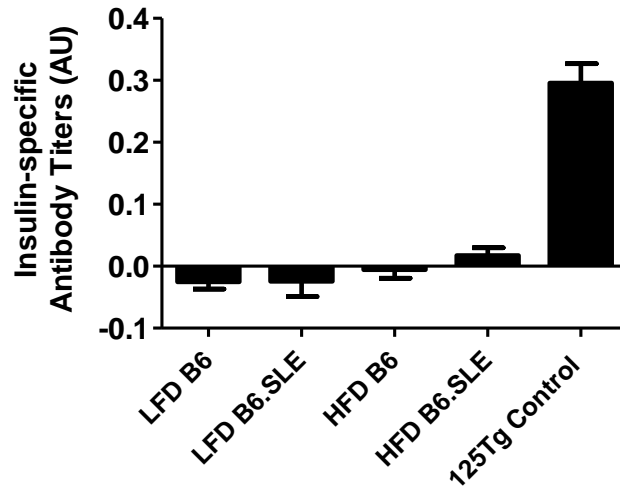


Figure 11. Insulin-specific IgG Antibodies Are Not In the Circulation of B6.SLE Mice. Male mice were fed HFD or LFD for 20 weeks and blood was collected via the retro-orbital sinus. A modified ELISA was used to detect insulin-specific antibodies in the serum of these mice. Data are expressed as the difference in absorbance between the uninhibited samples and the corresponding insulin-inhibited samples. n=3-10 mice/group.

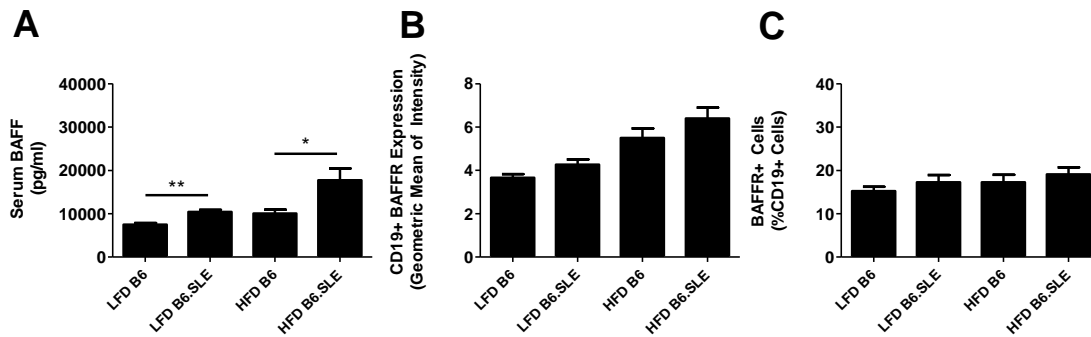
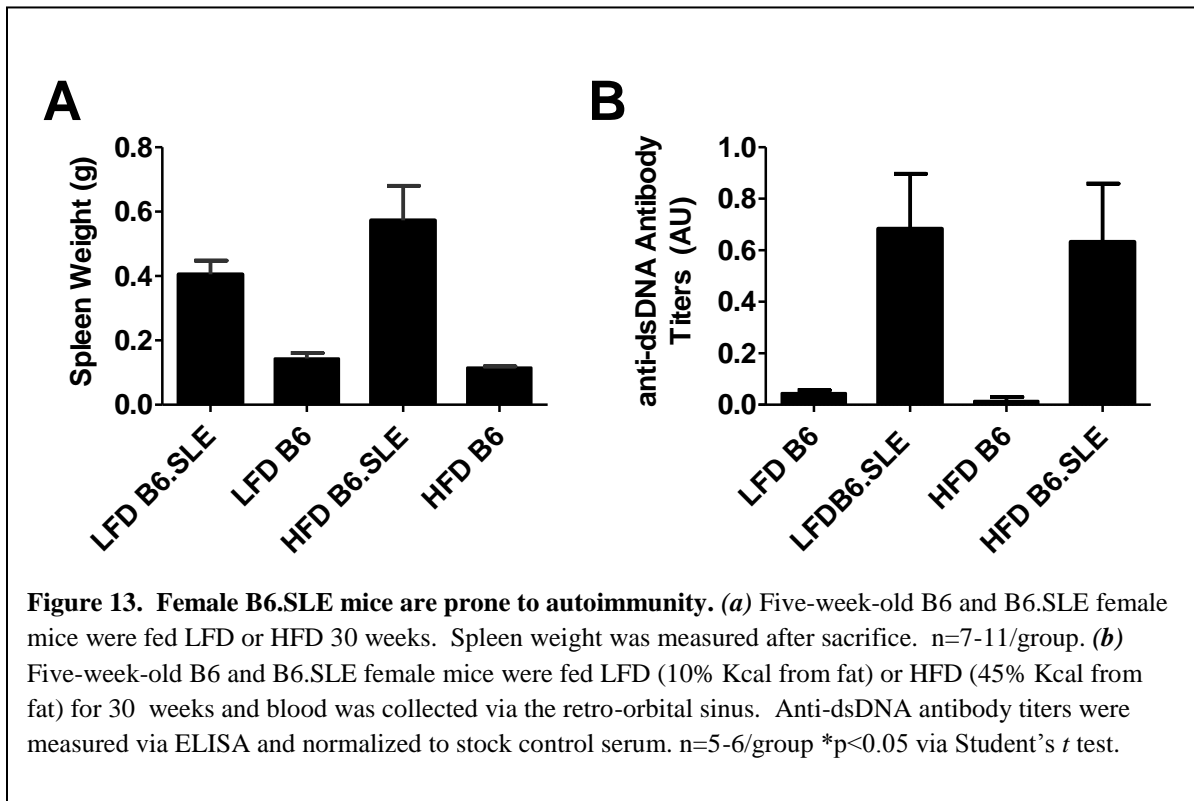


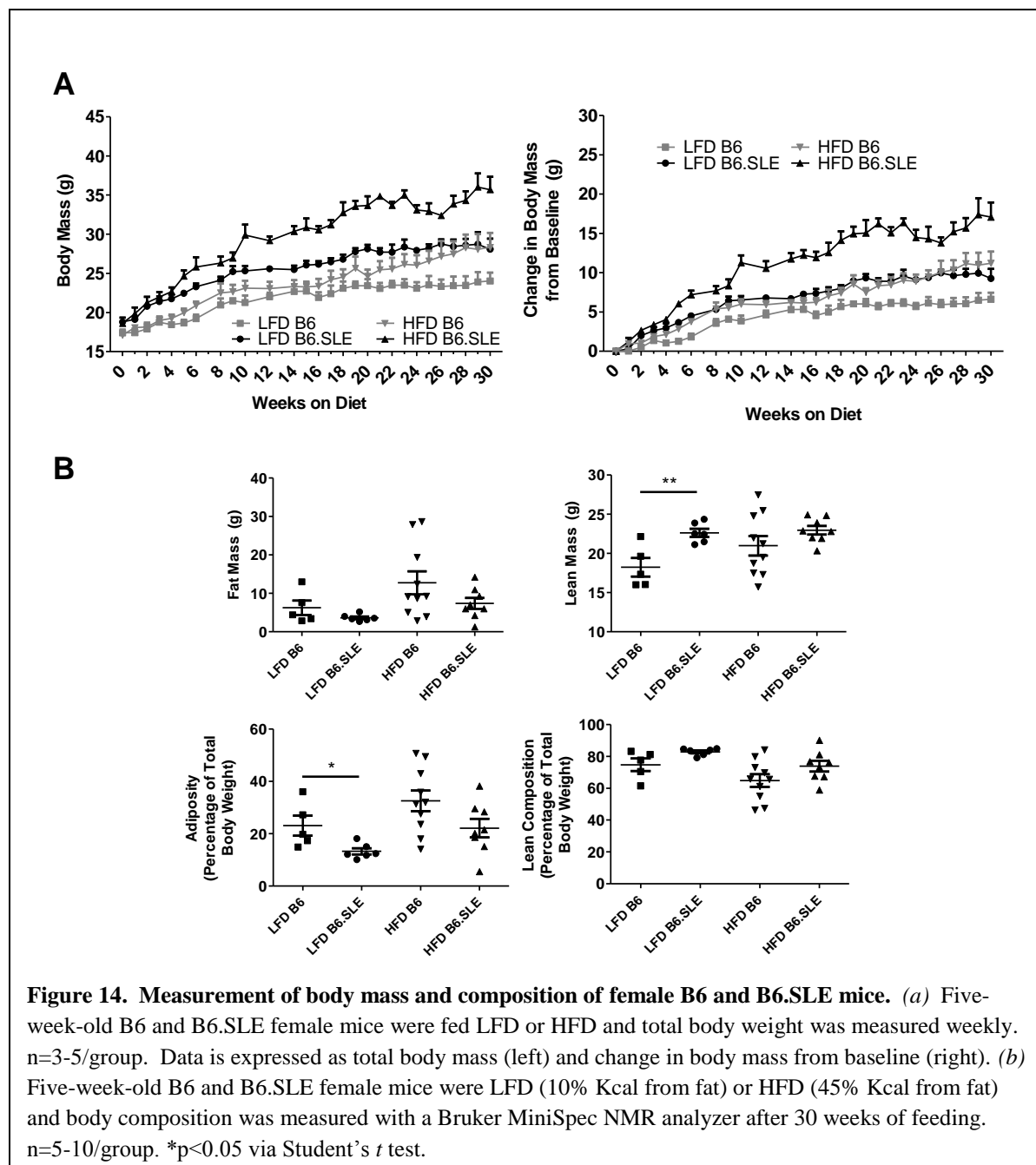
Figure 12. Measurement of BAFF and BAFFR. (a) Male mice were fed HFD or LFD for 16-25 weeks before sacrifice. Blood was collected via the retro-orbital sinus and serum BAFF was measured using conventional sandwich ELISA. n=6-8 mice/group. *p<0.05, **p<0.0007 by Student's *t* test. (b) Female mice were fed HFD or LFD for 30 weeks before sacrifice. SVF cells were stained and analyzed using conventional flow cytometry techniques. n=5-8 mice/group. *p<0.05 by Student's *t* test.

mice is due to anti-insulin antibodies. As seen in Figure 11, the titers of anti-insulin antibodies were not significantly different than that of control mice, indicating that the antibody detected in the adipose tissue may be causing effects through reacting with antigens other than insulin.

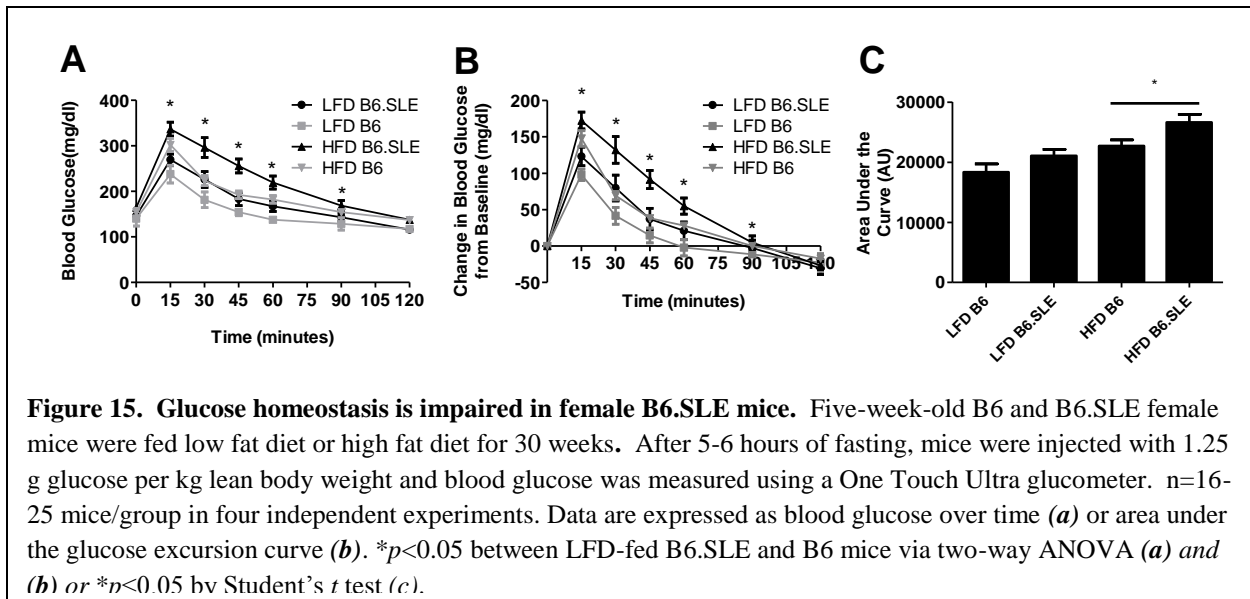
Overall, the data show that SVF B cells are skewed towards a pro-inflammatory IgG response, that circulating IgG1 levels are elevated in the serum of B6.SLE mice and that there is increased IgG deposition in the fat pads of B6.SLE mice.

Circulating BAFF is Increased in the Serum of B6.SLE Mice. B cell Activating Factor (BAFF) is a growth factor that promotes proliferation, maturation and antibody production of B cells. Elevated levels of circulating BAFF have been observed in the context of SLE and may be associated with increased disease severity and production of pathogenic autoantibodies (26). Furthermore, BAFF has been shown to impair insulin signaling in adipocytes *in vivo* and *in vitro* (80). To determine whether circulating BAFF levels are elevated in the B6.SLE strain, we collected serum from male mice fed diet for 16-25 weeks. BAFF levels were significantly elevated in LFD- and HFD-fed mice relative to their B6 controls (Figure 12). There was a trend towards higher BAFF levels in HFD-fed B6.SLE relative to LFD-fed B6.SLE mice, indicating





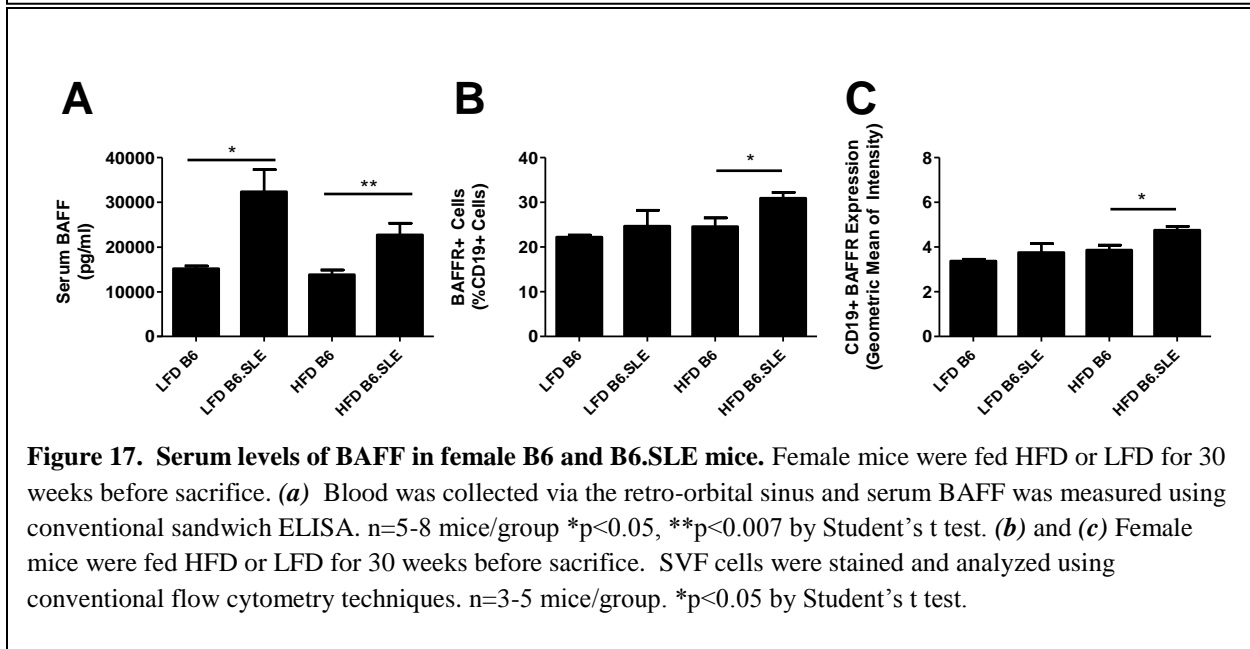
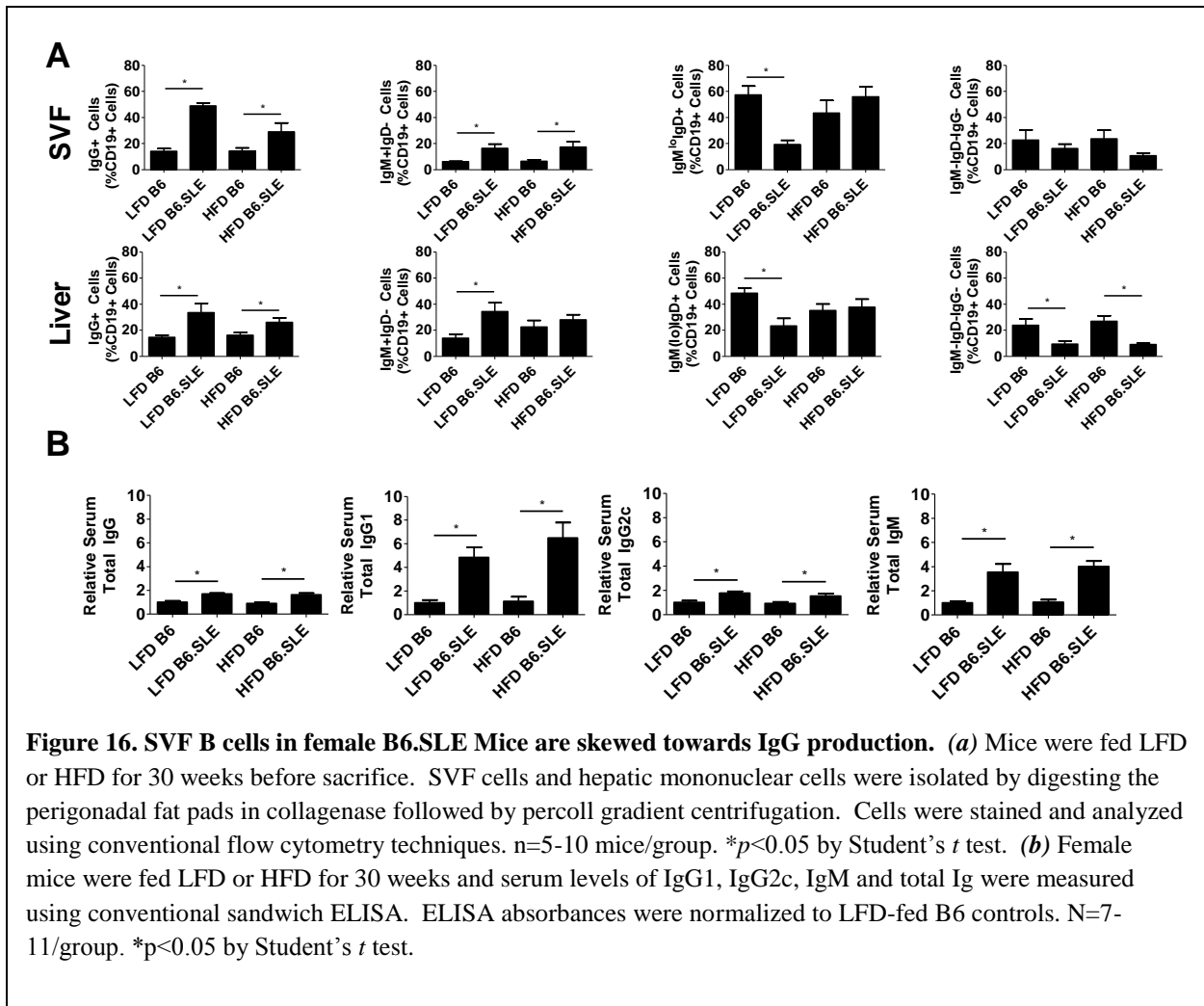
that HFD feeding may be associated with higher BAFF levels in these mice. BAFF receptor (BAFFR) is expressed on mature peripheral B cells and its ligation promotes cell survival through activation of Bcl-2 survival pathway (26). We used flow cytometry to determine whether SVF B cells from male B6.SLE mice had different levels of BAFFR expression than controls (Figure 12b-c). B6.SLE mice had trends towards higher BAFFR expression than B6



mice and LFD-fed B6.SLE mice and there were trends towards higher BAFFR expression in HFD-fed groups relative to their respective LFD-fed groups. Taken together, these data suggest an environment in which B cells in HFD-fed B6.SLE mice are predisposed to greater activation and survival due to higher levels of circulating BAFF and increased expression of BAFF receptor on SVF B cells.

Similarities and Differences in Metabolic Disease in Male and Female B6.SLE Mice

Female B6.SLE mice develop similar autoimmune disease to male mice and generate anti-dsDNA antibody titers and severe splenomegaly (Figure 13a-b). Metabolic disease was also assessed in female B6.SLE mice fed LFD or HFD for 30 weeks. Unlike male B6.SLE mice, female mice gain significantly more weight than their respective B6 controls when data is expressed as absolute body weight and change in weight from baseline (Figure 14a). LFD-fed B6.SLE mice had significantly higher lean body mass and a trend towards lower fat mass than controls. Similarly, HFD-fed B6.SLE mice had trends towards higher lean body mass and lower adipose tissue mass than controls (Figure 14b). Unlike male B6.SLE mice, significant worsened



glucose intolerance was seen in the HFD-fed B6.SLE group whereas there was only a slight trend toward higher glucose intolerance in the LFD-fed B6.SLE group (Figure 15). Like male mice, female B6.SLE mice had increased proportions of IgG class-switched B cells in the stromal vascular fraction (Figure 16a) and increased circulating IgG, IgG1, IgG2c and IgM relative to controls in both diet groups (Figure 16b). Furthermore, BAFF levels were significantly higher in B6.SLE relative to controls in both diet groups (Figure 17a) and there was significantly higher BAFF receptor expression on SVF-resident B cells in HFD-fed B6.SLE mice (Figure 17b-c).

Discussion

In this report, we show for the first time that B6.SLE mice develop more severe glucose intolerance and adipose tissue insulin resistance than B6 controls and that B6.SLE B cells have a diabetogenic immunophenotype characterized by increased circulating IgG, increased IgG expression on B cells and increased deposition of IgG heavy chain in white adipose tissue (80, 224). These changes were observed in LFD-fed B6.SLE mice, indicating that SLE disrupts glucose metabolism in the context of a low fat diet. Adiposity did not vary significantly between B6.SLE mice and their controls, indicating that lupus-related immune dysregulation—and not adipose tissue accumulation—causes disruption of glucose homeostasis in mice. Assessment of pancreatic islet function showed increased glucose-stimulated insulin secretion in B6.SLE islets. However, there was not a concomitant increase in fasting serum insulin in B6.SLE mice, which could be explained by either increased insulin clearance or lower islet cell mass. Estimation of insulin clearance with the C-peptide-to-insulin ratio (Figure 6a, Right Panel) showed no difference between mouse strains. Collectively, these data suggest that B6.SLE mice may be compensating for lower islet cell mass by increasing islet secretory capacity. Islet cell mass was

not measured, so we cannot confirm there is a defect in islet cell mass in B6.SLE mice in the present study.

These findings contrast with the earlier mouse studies of Ryan et al. (185) in the NZW/F1 model of lupus, in which NZW/F1 mice were shown to have hyperinsulinemia, hyperleptinemia and higher glucose intolerance than control parental strain mice. Our study differs from that of Ryan et al. in several key areas. Firstly, the NZW/F1 mice in *Ryan* et al. study reported significantly larger white adipose depots than controls. In some cohorts, NZW/F1 mice had twice as much visceral adipose tissue than controls, making it difficult to determine whether the metabolic differences observed were due to changes in body composition or the immune dysregulation observed in the setting of SLE. Our study shows that B6.SLE mice have decreased glucose tolerance and adipose tissue insulin resistance despite having similar adipose tissue mass and increased lean mass relative to controls. Secondly, given the newly characterized importance of the adaptive immune system in metabolic syndrome and the critical role of the adaptive immune system in the pathogenesis of SLE, our studies focused on the immunophenotype of the SVF lymphocyte compartment. We observed significantly increased percentages of T cells in the SVF of LFD-fed B6.SLE mice relative to controls. Although no differences in total B cell proportions were detected in B6.SLE mice on either diet, there were several qualitative differences in the B cell compartment. Specifically, the proportion of IgG-positive B cells was significantly increased in both diet groups and the activation marker CD80 was increased on B cells in LFD-fed B6.SLE mice. Circulating IgG was increased in the periphery of B6.SLE mice on both diets and there was a significant increase in IgG deposition in the adipose tissue of B6.SLE mice. These results are consistent with the diabetogenic B cell immunophenotype described by Winer et al. (224), in which IgG antibody secreted by B cells in

the context of HFD contributed to insulin resistance. Future studies will focus on whether passive immunization with IgG from LFD-fed B6.SLE mice is sufficient to induce glucose intolerance and adipose tissue insulin resistance in recipient mice.

Adipocyte hypertrophy in visceral adipose tissue has been associated with insulin resistance and hyperlipidemia. Although we observed increased adipocyte size in LFD-fed B6.SLE mice relative to controls, there were decreases in serum triglyceride and cholesterol in B6.SLE mice. We have previously observed reduced triglyceride and cholesterol levels in studies of B6.SLE mice (21, 199) and other groups have shown similar changes in serum lipids in patients with rheumatoid arthritis and other autoimmune diseases (88, 114, 183, 189, 204). The mechanism that causes this phenomenon is not known, but may result from disruption of lipid homeostasis by the chronic inflammation present in autoimmune disease (204).

Fasting levels of glucose, insulin, leptin, resistin, TNF α , and IL-6 were not significantly different between LFD-fed B6.SLE mice and their controls. This may indicate that the metabolic phenotype only becomes unmasked after challenge with glucose or insulin, or that the phenotype is only apparent in the fed state. Levels of glucose, insulin, leptin and resistin in the fed state have not been measured in the current study. Furthermore, we observed worsened glucose tolerance and adipose tissue insulin resistance in LFD-fed B6.SLE mice (Figure 4c) which was not accompanied by significant insulin resistance in liver or skeletal muscle. Adipose-tissue-specific impairment of insulin signaling has been shown to ameliorate glucose intolerance in fat-specific insulin receptor knockout (FIRKO) mice (16). Within this paradigm, we would not anticipate that the LFD-fed B6.SLE mice would have significantly worsened glucose tolerance. However, it is possible that non-classical, SLE-mediated effects on adipose tissue may be causing the observed metabolic impairment in B6.SLE mice. One molecule associated with both

SLE and impairment of adipocyte function is B cell activating factor (BAFF). We observed increased levels of circulating BAFF in B6.SLE mice, and SVF-resident B cells in these mice had elevated expression of the BAFF receptor. B cell Activating Factor (BAFF) is a growth factor that promotes proliferation, maturation and antibody production of B cells. Elevated levels of circulating BAFF have been observed in the context of SLE and is associated with increased disease severity and production of pathogenic autoantibodies (26). In addition, adipocytes have been shown to express the BAFF receptor and BAFF has been shown to specifically disrupt glucose homeostasis both *in vitro* and *in vivo* (80). Future studies will focus on the role of BAFF and IgG in the pathogenesis of metabolic disease in the context of B6.SLE mice.

Adiposity of B6.SLE mice did not differ significantly from that of B6 mice. Although early studies in humans showed higher body mass index (BMI) in cohorts of lupus patients (100, 101), subsequent studies failed to show significant differences in BMI between SLE patients and healthy controls (116, 167, 209, 210). In one of these studies, Magadmi *et. al* reported that SLE patients had significantly worsened insulin resistance than healthy control patients, but body composition did not differ significantly between SLE and control groups and SLE patients were only moderately overweight (BMI=26.6) (116). The observations of Magadmi *et al.* have been corroborated by several other groups (1, 27, 36). Therefore, we propose that the LFD-fed B6.SLE mice are a very suitable model of SLE-associated metabolic disease. Unexpectedly, reductions in glucose tolerance and insulin signaling were not observed in HFD-fed B6.SLE mice relative to B6 controls. It is possible that HFD feeding “saturates” the phenotype of B6.SLE mice, making it difficult to detect differences in glucose tolerance and insulin signaling between strains. Subsequent studies will focus on LFD-fed B6.SLE mice.

In conclusion, we have shown that B6.SLE mice fed LFD have significantly worsened glucose tolerance and adipose tissue insulin resistance than B6 controls. This B6.SLE glucose intolerance occurs in the presence of a diabetogenic B cell environment which is characterized by the presence of an increased number of IgG-producing B cells and IgG depositions in the white adipose tissue as well as higher levels of circulating IgG. The hyperglycemia and insulin resistance present in B6.SLE mice may contribute to CVD by causing increased expression of chemotactic factors and adhesion molecules by vascular endothelial cells and by causing ER stress-induced apoptosis in macrophages (20) Although it is clear that SLE patients and the B6.SLE mouse model suffer disproportionately from CVD (9, 199), the causes of this disparity have not been completely elucidated. Insulin resistance has higher prevalence in the SLE population and it is conceivable that it could be a contributing factor in the high CVD burden in these patients. Our current findings suggest potential mechanisms that give rise to metabolic disease in SLE patients and form the foundation for additional studies that could lead to greater understanding of the pathogenesis of SLE co-morbidities.

CHAPTER III

THE EFFECT OF HIGH FAT DIET ON THE PATHOGENESIS OF LUPUS IN B6.SLE MICE

Introduction

Systemic lupus erythematosus (SLE) is characterized by the production of autoantibodies against self-antigens such as, double stranded DNA (dsDNA) and phospholipids. Unfortunately, the etiology and environmental triggers for SLE remain largely unknown. Therefore, it is necessary to complete studies of variables that increase personal risk for development and exacerbation of the progression of SLE and its comorbidities.

A CDC Morbidity and Mortality Weekly Report published in 2002 showed that the SLE death rate increased by 33% between 1979 and 1998. Although this expansion of the lupus patient population could have resulted from improved diagnostic techniques, it could also be due to an actual increase in the incidence of SLE and/or more severe disease secondary to environmental factors. One environmental factor that has rapidly increased during this time period is obesity. The epidemic of overweight and obesity is the preeminent health concern of the Western world and is associated with a constellation of comorbidities, including dyslipidemia, hypertension and insulin resistance. In addition to these classical comorbidities, there is increasing evidence that obesity negatively impacts a wider spectrum of diseases such as cancer and inflammatory diseases. Because SLE has been demonstrated to be exacerbated by high fat diet in both humans (37, 116) and mouse models (5, 44, 98, 110, 143, 240) and recent studies have demonstrated that SLE alters insulin sensitivity, it is important to investigate how metabolism and autoimmunity are interlinked.

Several studies have linked diet and obesity to exacerbation of autoimmunity. When lupus prone NZB/W mice were fed a high-fat, lard-based diet, they had higher mortality rates and more severe glomerulonephritis than mice fed a low-fat diet (98), and a diet high in omega-3 polyunsaturated fatty acids protected against disease (5, 77). High fat diet also worsens disease in the MRL/lpr mouse model of lupus (143). These effects are not limited to lupus: HFD feeding exacerbates other mouse models of disease colitis and experimental autoimmune encephalomyelitis secondary to diet-mediated changes to changes in the T lymphocyte compartment (226). Furthermore, diet supplementation with omega-3 polyunsaturated fatty acids ameliorates disease in humans with SLE (217).

Patients with lupus are nearly eight times more likely to die of renal disease than the general population (14) and the majority of kidney disease burden consists of diffuse proliferative lupus nephritis and membranous lupus nephritis (132). Lupus nephritis is instigated when anti-nuclear antibodies form immune complexes which deposit on the glomerular basement membrane. This spurs the activation of a signaling cascade of chemokines and pro-inflammatory cytokines which directly damage the glomerulus and indirectly cause damage through leukocyte recruitment (123, 245). T cell infiltrates include Th1, Th2 and Th17 subtypes (132), and B cell infiltrates occupy the renal tubulointerstitium where they produce anti-nuclear antibodies (57). SLE patients have higher circulating TH17 cell numbers and higher plasma levels of IL23 and IL-17 (229) and these results are mirrored in the MRL/lpr mouse model of lupus (106, 244). Furthermore, IL-23R deficiency prevented anti-dsDNA antibody production and protected against the development of glomerulonephritis in this model (106, 244). Kidney damage in both human and mouse studies was attributed in part to kidney infiltration by an IL-

17-producing lymphocyte population that was enriched for CD4-CD8- double negative (DN) T cells that produce IL-17, IFN- γ , and TNF- α (46).

Interestingly, HFD and obesity exacerbate disease in mouse models of colitis and multiple sclerosis through expansion and activation of the Th17 population (226) and it is possible that HFD influences the development of lupus nephritis in a mouse model of lupus through similar mechanisms. To test this hypothesis, we fed LFD or HFD to the B6.SLE mouse model of lupus, which was bred to have fully-penetrant glomerulonephritis (139) and measured parameters of renal function, morphology and inflammatory state. Although significant worsening of these parameters was observed in B6.SLE mice relative to B6 controls, HFD-fed B6.SLE mice did not have a worsened kidney disease relative to their LFD-fed counterparts.

Materials and Methods

Mice. B6.*Sle.1.2.3* mice, hereafter referred to as B6.SLE mice, were a generous gift of Edward Wakeland (UTSW, Dallas, TX) and have been previously described (139). C57BL/6 mice (B6) were originally purchased from Jackson Labs (Bar Harbor, ME), and were bred in the animal facility at Vanderbilt University Medical Center. All procedures involving animal subjects received prior approval from the Vanderbilt University Institutional Animal Care and Use Committee.

Diet. B6 and B6.SLE mice were co-housed after weaning and fed low fat diet (LFD, 10% Kcal from fat, D12450B, Research Diets, New Brunswick, NJ) or HFD (HFD, 45% Kcal from fat, D12451, Research Diets, New Brunswick, NJ) *ad libitum*.

Flow Cytometry. Single-cell suspensions of kidney mononuclear cells were isolated as follows: kidneys were minced and digested for 20 minutes at 37° C with 1mg/ml collagenase in HBSS (Sigma, St. Louis, MO). Digested kidney was passed through a 40 micron filter and

mononuclear cells were isolated from the interface of a 40%/60% Percoll gradient (GE Healthcare, Piscataway, NJ). In order to minimize non-specific binding of flow antibodies, cells were blocked for 15 minutes at RT with a 1:200 dilution of Fc receptor block (BD Biosciences, San Diego, CA) in a FACS buffer consisting of 1X HBSS, 1% BSA, 4.1663 mM sodium bicarbonate and 3.0765 mM sodium azide. The following fluorescently-labeled antibodies were diluted 1:200 in FACS buffer and incubated with the cells for 45 minutes at 4° C: CD19-PECy7, B220-A450, TCR β -PECy7, CD8-FITC and CD4-APCCy7 (BD Biosciences, San Diego, CA). The labeled cells were washed 3 times with FACS buffer and fixed overnight at 4° C in a solution of 2% paraformaldehyde in PBS. Labeled cells were analyzed on a MACSquant seven-color flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) and data was analyzed with FCS Express 4 (De Novo Software, Los Angeles, CA).

Real Time. Kidney RNA was isolated using a RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The quantitation assays were completed using Taqman Gene Expression Master Mix (Applied Biosystems, Foster City, CA) and Taqman Probes for the specified genes according to manufacturer's instructions. Threshold Ct values were measured with SDS2.3 software (Applied Biosystems, Foster City, CA) and transcript levels measured using the $2^{-\Delta\Delta Ct}$ method. 18s was used as the reference gene and the LFD-fed B6 group was used as a comparator.

Histology. Female mice were sacrificed after 26 weeks of HFD feeding and kidneys were removed, embedded in paraffin and stained with H&E. Glomerulus size was measured in 4-5 independent fields per slide using ImageJ software (National Institutes of Health, Bethesda, MD) and data was expressed as pixels per glomerulus.

Measurement of Serum Creatinine and Urine Protein. Male B6 and B6.SLE mice were fed HFD or LFD for 20 weeks. Serum was collected and creatinine concentration was measured using a QuanticChrom creatinine assay kit (BioAssay Systems, Hayward, CA) according to manufacturer's instructions. Urine protein was measured in randomly sampled urine from study mice using Multistix 10SG urinalysis strip (Siemens, Malvern, PA).

Statistics. Statistical significance for GTT data was calculated with a two-way ANOVA for repeated measures. All other significance was calculated with a Student's *t* test comparing mice within diet groups. All calculations were completed with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Unless otherwise noted, all data was expressed as mean +/- SEM.

Results

Glomerular Tuft Size is Not Influenced by High-Fat Diet Feeding. We assessed glomerular morphology in order to determine whether HFD exacerbated kidney disease in B6.SLE mice. Overall, B6.SLE mice in both diet groups had focal to diffuse proliferative changes that consisted of increased cellularity composed of endothelial and mesangial cells, increased mesangial matrix, thickening of capillary walls and areas of necrosis (Figure 18). Glomerular tuft size is correlated with renal injury and is increased in B6.SLE mice relative to B6 controls. Both HFD-fed and LFD-fed mice had equal glomerular tuft size indicating that diet does not influence this disease parameter.

Kidney mass was also measured in LFD- and HFD-fed B6 and B6.SLE mice (Figure 19). Total kidney mass was increased in HFD-fed B6.SLE mice relative to the B6 control group but there was no difference in kidney mass between the LFD-fed groups. Additionally, there was a trend towards higher mass in the HFD-fed B6.SLE mice relative to their LFD-fed B6.SLE counterparts.

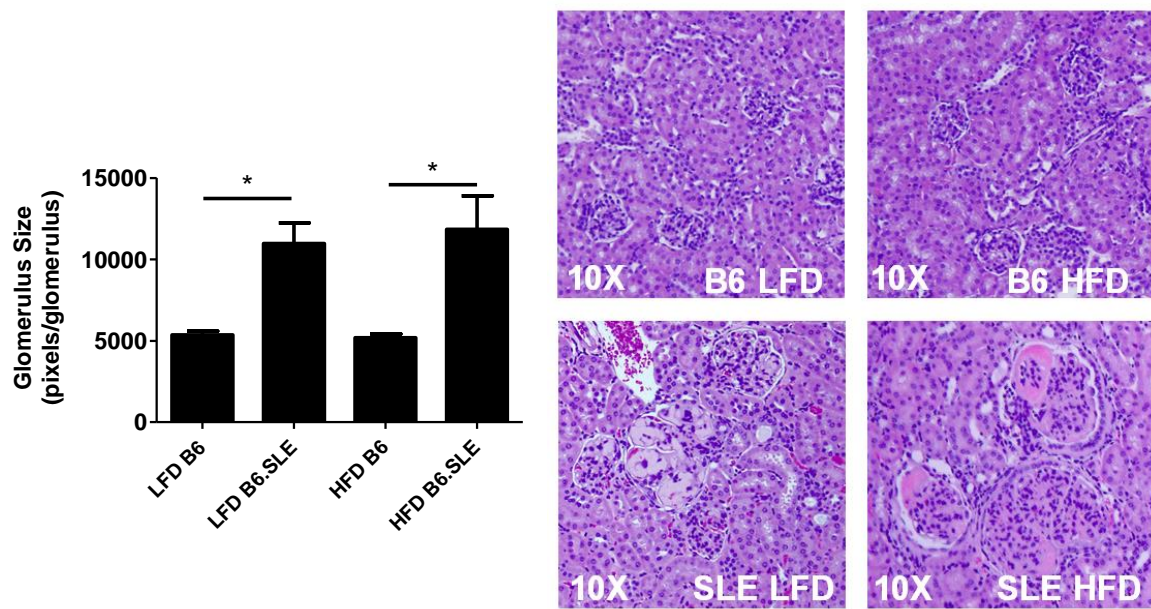


Figure 18. Glomerular tuft size is increased in B6.SLE mice. Female mice were sacrificed after 26 weeks of HFD feeding and kidneys were removed, embedded in paraffin and stained with H&E. Glomerulus size was measured in 4-5 independent fields per slide and data was expressed as pixels per glomerulus. (n=4-5 per group. *p<0.05).

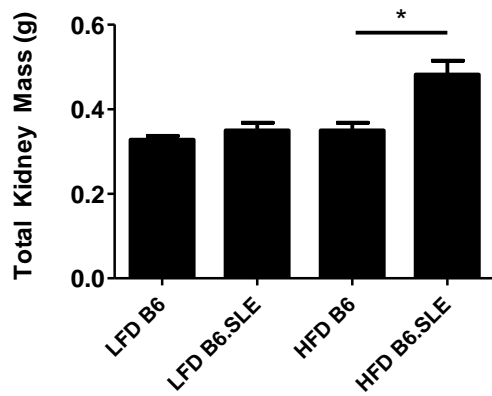


Figure 19. Kidney weight is increased in HFD-fed B6.SLE mice. Female B6 and B6.SLE mice were fed HFD or LFD for 28 weeks. Mice were sacrificed and kidneys were weighed. Data is expressed as the total weight of both kidneys. n=4-6 mice/group. *p<0.05.

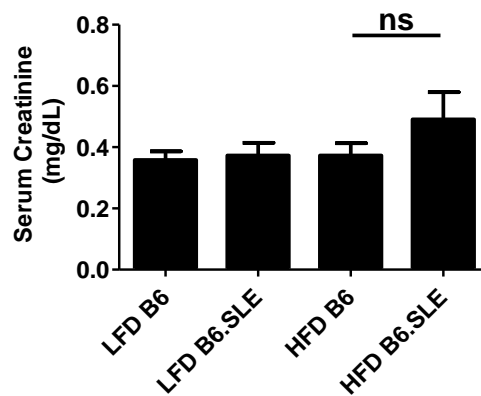


Figure 20. Serum creatinine is not increased in B6.SLE mice. Male B6 and B6.SLE mice were fed HFD or LFD for 20 weeks. Serum was collected and creatinine concentration was measured using the Jaffe picrate method. n=5-7 mice/group.

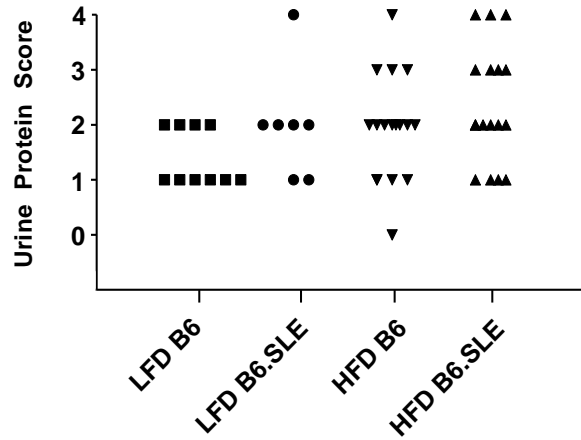


Figure 21. Urine protein trends higher in HFD-fed B6.SLE mice. Male B6 and B6.SLE mice were fed HFD or LFD for 20-28 weeks. Urine was sampled from mice and applied to a Siemens Multistix 10SG urinalysis strip. Results are expressed as urine protein score, with higher numbers correlating with higher urine protein content.

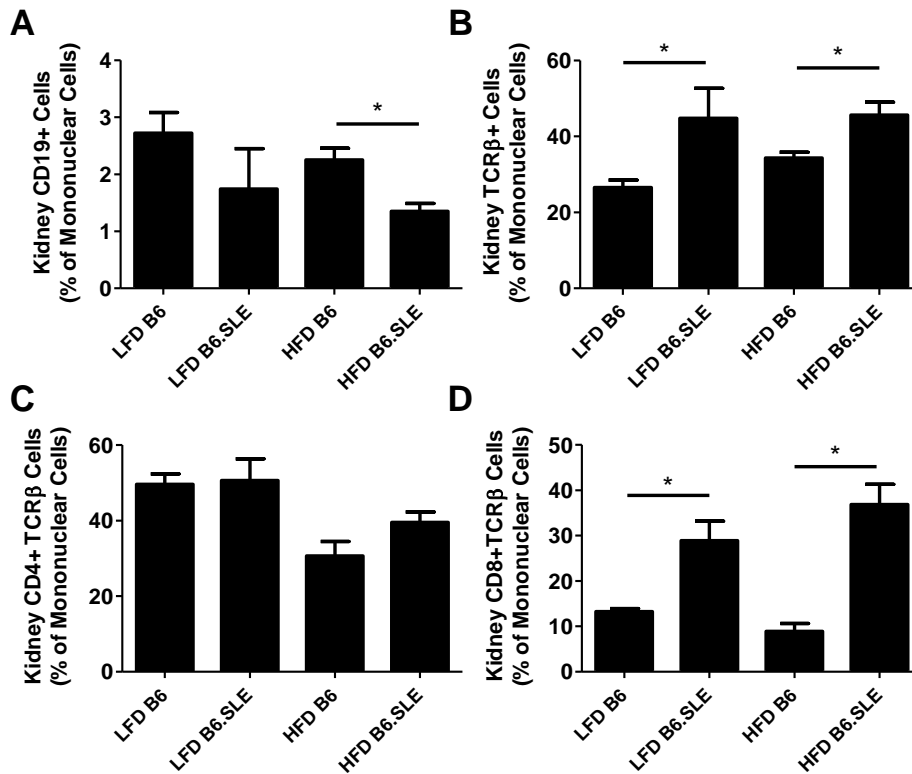
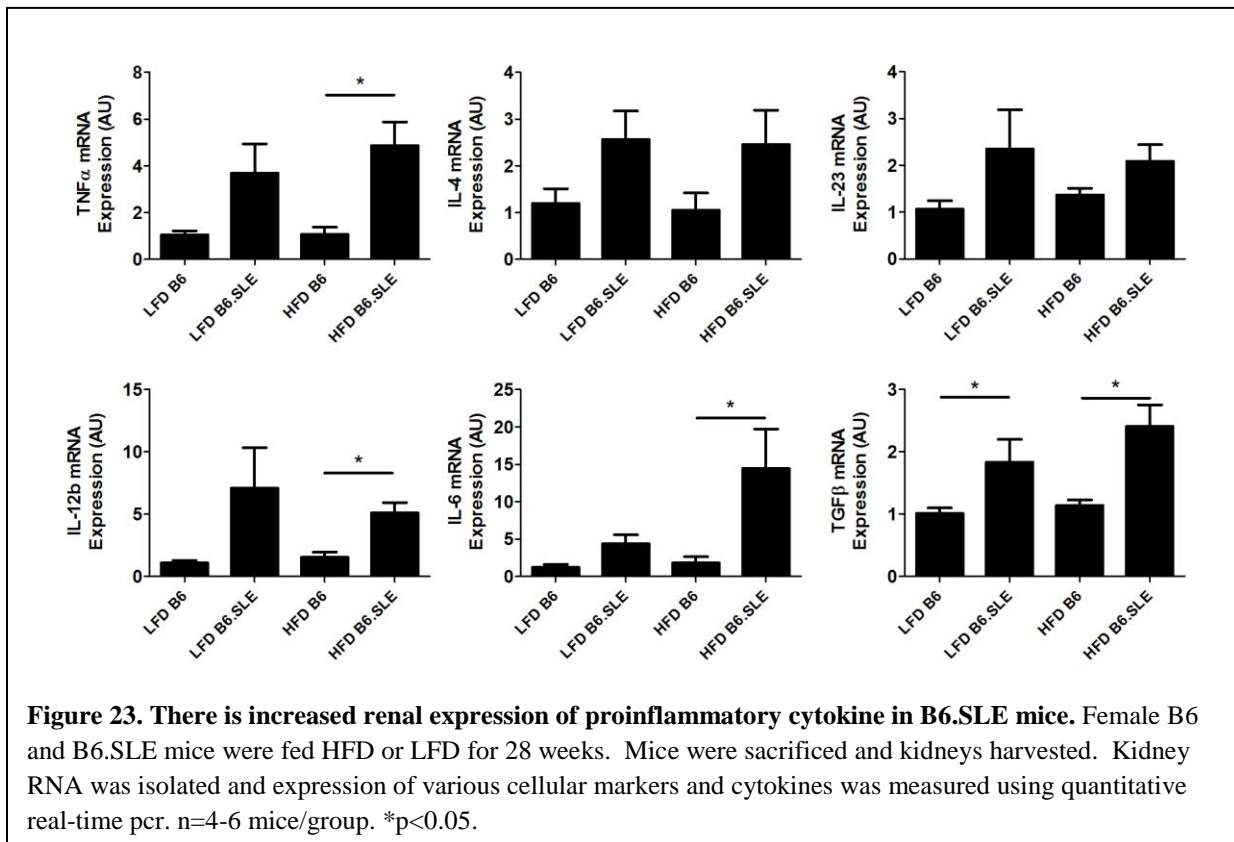


Figure 22. The CD8+ T cell population is increased in B6.SLE mice. Female B6 and B6.SLE mice were fed HFD or LFD for 28 weeks. Mice were sacrificed and kidneys harvested. Kidney mononuclear cells were isolated using a Percoll gradient and cells were stained for markers of B cells and T cells. n=4-6 mice/group. *p<0.05.

Kidney Function is Not Significantly Worse in B6.SLE Mice. Serum creatinine and urine protein levels positively correlate with worsened kidney function. There were no significant differences in serum creatinine between any groups (Figure 20), but there was a trend towards higher creatinine in HFD-fed B6.SLE mice. There were also trends towards higher urine protein in B6.SLE relative to B6 controls, and trends towards higher urine protein in HFD-fed groups relative to their LFD-fed counterparts (Figure 21).



HFD Does Not Significantly Influence Inflammation or the Immune Cell Compartment in B6.SLE mice. The kidney mononuclear cell population was measured using flow cytometry (Figure 22). There was a significant increase in the proportion of TCR β ⁺ T cells in B6.SLE groups which was accompanied by a relative decrease in kidney CD19⁺ B cells. The CD8⁺ T cell population constituted most of the increase in renal T cells. There was a significant increase

in expression of the pro-inflammatory cytokines TNF- α , IL-12b, IL-6 and TGF β in HFD-fed B6.SLE mice and trends towards higher expression of these cytokines in the LFD-fed B6.SLE mice, indicating a trend towards higher inflammation in the kidneys of SLE-prone mice (Figure 23).

Discussion

We hypothesized that HFD would exacerbate kidney disease in the B6.SLE mouse model of lupus. Several prior studies have indicated that HFD feeding exacerbates disease in mouse models (5, 44, 98, 110, 143, 240) and in humans (37, 116) and also worsened mouse models of EAE and colitis. To test this hypothesis, we fed HFD to lupus-prone B6.SLE mice and measured kidney morphology, renal function, kidney cellular content and renal inflammation. Although B6.SLE mice had significantly worsened kidney inflammation and more T cell infiltrate than B6 controls, these parameters were nearly equal between HFD-fed B6.SLE and LFD-fed B6.SLE mice.

There are several factors that could have contributed to the lack of diet response in HFD-fed B6.SLE mice. This is the first study of dietary effects on lupus nephritis in the B6.SLE mouse model. Previous studies primarily focused on the NZB/NZW F1 or MRL/*lpr/lpr* models which may be more susceptible to HFD-feeding than B6.SLE mice. Furthermore, although the percentage of lard by weight was similar between the diets used in our studies and previous studies, it is possible that differences in other macronutrients and micronutrients (not described in the original publications) influenced disease levels. The length of our experiment was roughly equivalent to those of previous experiments, but it is possible that differences may be observed if the experimental duration was extended in this mouse model. Furthermore, a diet higher in fat might be necessary to elicit changes in B6.SLE mice. One study showed that lupus-prone mice

fed a diet high in omega 3 polyunsaturated fatty acids had less severe disease than mice fed a lard-based diet. It is important to repeat these studies in B6.SLE to determine whether polyunsaturated fatty acids could also ameliorate disease in this strain.

In conclusion, B6.SLE mice fed a high-fat diet (45% Kcal from fat) did not have significantly worsened kidney disease compared to LFD-fed B6.SLE mice as measured by kidney morphology, renal function, kidney cellular content and renal inflammation. Although the 45% Kcal from fat diet did not exacerbate disease, it will be necessary to repeat this experiment with a higher-fat diet (60% Kcal from fat) or a diet high in omega 3 polyunsaturated fatty acids in order to determine whether these diets worsen or ameliorate disease.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Comparison of Metabolic Disease in the B6.SLE Strain to Humans with SLE

Humans suffering from SLE are at increased risk of developing insulin resistance and other components of the metabolic syndrome. Although SLE patients and B6.SLE mice both have similar adiposity to healthy controls (10, 38, 116), the manifestation of metabolic disease in B6.SLE mice differs from SLE patients in several ways. SLE patients have elevated fasting insulin levels whereas insulin levels in B6.SLE mice are equivalent to controls, and both SLE patients and B6.SLE mice maintain normoglycemia in the setting of lupus (37, 38, 210). Most studies of human SLE patients showed that there was no increase in total serum cholesterol (38, 116, 167, 189, 210) and at least three separate studies showed that serum triglycerides were increased in the SLE patient population (37, 38, 116). In contrast, B6.SLE mice had significantly lower total serum cholesterol and triglyceride than B6 controls in both diet groups. All known studies of metabolic disease in SLE patients have been completed in females, owing to the relative scarcity of male SLE patients. The studies in Chapter 2 included both male and female B6.SLE mice. We found that LFD-fed male B6.SLE mice and HFD-fed female B6.SLE mice had increased glucose intolerance that was independent of body composition. Male mice and humans tend to be at higher risk of developing insulin resistance (122), and it is therefore possible that male SLE patients would have more severe insulin resistance than females with SLE.

The mechanisms that give rise to insulin resistance in the setting of human SLE have not been completely elucidated, although adiposity (116) and corticosteroid treatment (116, 167,

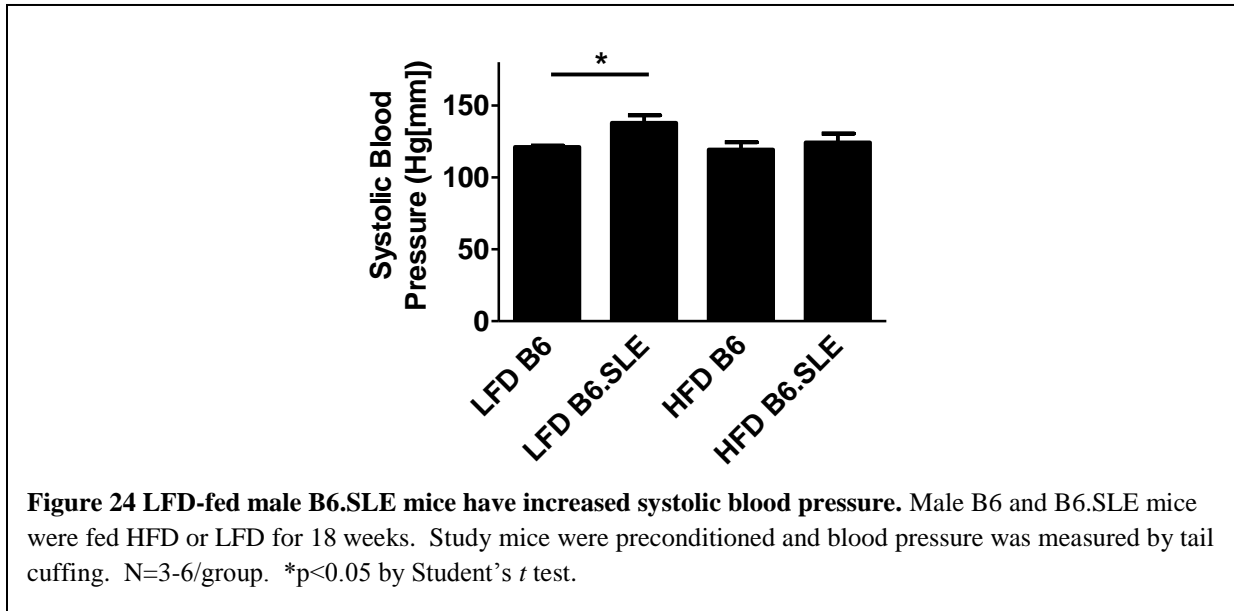
210) have been ruled out as contributing factors. The level of circulating IL-18, a pro-inflammatory cytokine which promotes the activation of T cells and NK cells, positively correlates with insulin resistance in SLE patients (85) and the general population (208) and may be a potential therapeutic and investigative target for SLE-associated insulin resistance. IL-18 levels were not measured in B6.SLE mice in our studies and it is not clear whether this cytokine contributes to insulin resistance and glucose intolerance in this strain. Another factor that may link SLE and insulin resistance is BAFF, which is described in the mechanism section below.

In conclusion, the B6.SLE strain mirrors some aspects of insulin resistance in humans with SLE. Both adipose tissue insulin resistance and glucose intolerance in B6.SLE mice occur in a body composition-independent manner, while SLE patients are hyperinsulinemic in the setting of normal BMI. Other aspects of metabolic disease in SLE patients, including hypertriglyceridemia, hyperinsulinemia and normocholesterolemia, and are not replicated in the B6.SLE model. The metabolic phenotype of the lupus-prone NZBWF1 strain, described by Ryan et al., has many similarities to the human population; however, it is unclear whether lupus disease contributed to metabolic disease in this model. The study of Ryan et al. will be compared to our study in the following section.

Comparison of Metabolic Disease in B6.SLE Strain to the NZBWF1 STRAIN

To our knowledge, there is only other study of IR and glucose intolerance in a mouse model of SLE. In that study, Ryan et al. examined glucose homeostasis in the NZB/W F1 strain, a progenitor of the B6.SLE strain (185). Similar to the results observed in B6.SLE mice, the NZB/W F1 strain had increased glucose intolerance in the setting of a low-fat, normal chow diet. Additionally, NZB/W F1 mice had increased mean arterial pressure relative to controls which correlates with the increased systolic blood pressure observed in LFD-fed B6.SLE mice (Figure

24). In contrast, Ryan et al. observed hyperleptinemia, hyperinsulinemia, and increased macrophage content in the white adipose tissue. This differs from LFD-fed B6.SLE mice, which had similar fasting insulin, fasting leptin and adipose tissue macrophage levels to B6 controls. Furthermore, there was increased hepatic lipid accumulation in NZB/W F1 mice, which was in contrast to the significantly reduced triglyceride content observed in the B6.SLE strain.



The discrepancies between our results and those of Ryan et al. were somewhat unexpected, as the NZB/W F1 strain is a progenitor of the B6.SLE strain. The NZB/W F1 strain is the first filial offspring (F1) of a mating between a New Zealand Black (NZB) female mouse and New Zealand White (NZW) male mouse. The offspring develop fully-penetrant glomerulonephritis which develops earlier and more severely in females (104). A series of crosses between NZB/W F1 mice, NZW mice and their offspring resulted in the generation of the New Zealand Mixed (NZM) 2410 strain, which also invariably develops fulminant lupus. Lupus susceptibility loci were genetically mapped in the NZM2410 strain (139) and these loci were backcrossed onto the C57BL/6 background to generate the B6.SLE strain (141). As in the

progenitor strains, both males and females in this strain suffer from fully-penetrant lupus and females tend to develop clinically apparent glomerulonephritis at a younger age. Lupus susceptibility in the NZB/W F1 and B6.SLE mouse models relies on multiple genes and both models mirror the polygenic nature of most forms of human SLE. However, one strength of the congenic B6.SLE strain relative to the NZB/W F1 strain is that their B6 background makes it possible to isolate the effects of the lupus susceptibility loci through the B6 controls. Only the minimal genetic intervals necessary to confer glomerulonephritis were backcrossed onto the C57BL/6 background in the B6.SLE strain, lessening the risk of disrupting genes necessary for normal glucose homeostasis in the C57BL/6 strain. However, the gene encoding the leptin receptor is present on the SLE2 locus on chromosome 3 and was carried over to the C57BL/6 background when the congenic B6.SLE strain was generated. LFD-fed B6.SLE mice had similar fasting leptin levels to B6 controls and HFD-fed B6.SLE mice had significantly lower fasting leptin than controls (Figure 6). This data provides preliminary evidence that normal leptin signaling is not impaired in B6.SLE mice, but no formal assessment of leptin receptor function was completed in these studies. Both the NZB parental strain or NZW parental strain may be used as controls when working with NZB/W F1 mice. In the studies of Ryan et al., the authors reported results from the NZW strain, but not the NZB parental strain. NZW mice frequently develop anti-ssDNA antibodies but their autoimmune disease is not clinically apparent at 36 weeks of age (104). NZB mice have more consequential disease than the NZW strain and produce anti-nuclear antibodies which can give rise to mild glomerulonephritis that becomes clinically apparent well after 36 weeks of age (83). Although body composition is comparable between NZB and NZW mice at 16 weeks of age (174), the body composition of these strains at 36 weeks of age has not been reported. Therefore, it is possible that levels of adiposity and

metabolic disease in NZB mice, but not NZW mice, are equivalent to those of the NZB/W F1 strain at 36 weeks of age. Further studies comparing NZB/W F1 mice to the NZB parental strain should be completed in order to determine whether glucose intolerance is secondary to the autoimmune disease present in NZBWF1 mice or SLE-independent accumulation of adipose tissue.

The studies of Ryan et al. focused on LFD-fed 36-week-old females, whereas we examined both 25-week-old male and 35-week-old female B6.SLE mice. Although we observed glucose intolerance in low-fat diet fed male B6.SLE mice relative to controls, no exacerbation in glucose tolerance was observed in LFD-fed female B6.SLE mice. However, glucose intolerance was significantly worsened in HFD-fed female B6.SLE mice relative to controls. Since female mice in our study were at a similar age to female mice in the Ryan study (35 weeks and 36 weeks, respectively), we expected LFD-fed female B6.SLE mice and NZB/W F1 mice to have equally high glucose intolerance. However, it is difficult to compare the results of Ryan et al. to ours, as NZB/W F1 body composition was radically different than that observed in B6.SLE mice. NZB/W F1 mice were over 11 grams heavier than controls and had twice as much visceral adipose tissue. Additionally, the total body weight and visceral adipose tissue depot mass were significantly higher than that observed in B6.SLE mice at the same age. LFD-fed B6.SLE mice weighed an average of 27 grams (versus 45.7 grams in NZB/W F1 mice) and had a total of 3 grams body fat (versus 5 grams of fat in the viscera alone in NZB/W F1 mice). These large differences in weight and adiposity complicate the interpretation of the results of Ryan et al. The overall phenotype in NZB/W F1 mice is more consistent with phenotype observed in obese C57BL/6 mice. Like obese mice, NZB/W F1 mice have hyperinsulinemia, hyperleptinemia, increased glucose intolerance, increased adipose tissue macrophage infiltration and increased

hepatosteatosis. In contrast, B6.SLE mice have similar or lower adiposity and significantly higher lean body mass than B6 controls in both genders and diet groups. Unlike obese mice, glucose intolerance and adipose tissue insulin resistance is not accompanied by hyperinsulinemia, hyperleptinemia, hepatic insulin resistance or hepatosteatosis. Therefore, we hypothesize that the mechanisms which contribute to the severe glucose intolerance observed in B6.SLE mice are distinct from those that give rise to glucose intolerance in obese mice or NZB/w F1 mice. Furthermore, since the NZB/W F1 strain is merely a hybrid of the parental NZB and NZW strains and parental genes unrelated to lupus pathogenesis are conferred to their offspring, it is possible that the metabolic results reported in Ryan et al. are not directly related to the pathogenesis of lupus. Additionally, the weight gain and adiposity observed in NZB/W F1 mice was likely exacerbated by the significantly lessened locomotor activity in this strain relative to the NZW controls.

In conclusion, we assert that the B6.SLE strain is more suitable than the NZB/W F1 strain as a model SLE-associated insulin resistance in humans. Although the manifestation of metabolic disease in the B6.SLE model varies from that of humans in regards to fasting insulin levels and serum triglyceride levels, both SLE patients and B6.SLE mice suffer disruption of glucose homeostasis in the setting of unchanged body composition. Although Ryan et al. observed significant hyperinsulinemia and glucose intolerance in the NZB/W F1 model, it is unclear whether metabolic changes are secondary to increased adiposity or lupus disease. Furthermore, the B6.SLE mouse is a more precise model than the NZB/W F1 strain, as only the lupus susceptibility loci were backcrossed onto the C57BL/6 background. Since NZB/W F1 mice are a hybrid of two different parental strains, it is difficult to design appropriate controls which isolate the influence of SLE susceptibility on metabolism from the general metabolic

characteristics of the parental strains. Therefore, future studies in this area should focus on the B6.SLE strain in order to gain a better understanding of the pathogenesis of metabolic disease in the context of SLE.

Proposed Mechanism

In chapter 2, we showed increased glucose intolerance and adipose tissue insulin resistance in B6.SLE mice. Interestingly, this phenotype was observed in the context of LFD-feeding and there was no significant difference in adiposity between B6.SLE and control B6 mice. To our knowledge, this is the first study that has shown spontaneous glucose intolerance and adipose tissue insulin resistance in LFD-fed mice which were not associated with changes in adiposity or deliberate modification of metabolism-related genes. Since there were no changes in body composition that would predispose mice to glucose intolerance, it is possible that SLE-associated immune changes are a key driver of dysfunctional glucose homeostasis. The influx of immune cells into the adipose tissue of B6.SLE mice differs slightly from that observed in the adipose tissue of obese mice. CD8⁺ T cell and macrophage proportions are increased in the adipose tissue of obese mice; although we observed increased CD8⁺ T cell proportions in B6.SLE mice, we did not observe a concomitant increase in macrophages (151, 225). Winer et al. showed an increase IgG deposition and IgG⁺ B cell content in the adipose tissue of HFD-fed mice, a phenomenon that was also present in B6.SLE mice (Figure 10).

Presumably, the adaptive immune system is responding to adipose tissue antigens which are revealed as part of obesity or the SLE disease process, but there is only tenuous evidence to support this hypothesis. There are no data showing that the adipose antigenic targets of the immune system are conserved between obesity-associated insulin resistance and SLE-associated insulin resistance, or even that targets are shared among all mice with obesity-associated insulin

resistance. However, the identification of adipose tissue target antigens would be a high-impact finding that could lead the development of therapeutics that could be used to treat type 2 diabetes in humans. Furthermore, it is possible that characterization of the adipose targets in SLE-associated insulin resistance could lead to the identification of antigen targets which would be applicable in obesity.

Lupus is characterized by the generation of antibodies against a wide variety of intracellular antigens, including dsDNA, histone, cardiolipin, and ribosomal proteins (148). These intracellular antigens become exposed during the process of apoptosis (215). Defects in the pro-apoptotic pathway components *lpr* and Bim have been observed in the SLE patient population and mice bearing these mutations suffer from early and severe SLE-like disease (87). Furthermore, clearance of apoptotic cells is impaired in both lupus patients and the NZB×NZW(F1) and MRL/*lpr* mouse models of lupus (81, 108, 146). The abnormal presentation of intracellular self-antigens, paired with defects in the negative selection of self-reactive lymphocytes (17), leads to the production of the anti-self-antibodies that are a hallmark of SLE.

Adipocyte apoptosis and necrosis have been observed in obese mice and humans and is associated with adipose tissue leukocyte infiltration and insulin resistance. HFD-fed mice lacking the pro-apoptotic factor Bid are protected against glucose intolerance and insulin resistance and have reduced adipose tissue macrophage infiltration (6). Crown-like structures, a key histological feature of obese adipose tissue that consists of leukocyte infiltrate, surround these dead or dying adipocytes and only 20% of baseline adipocytes remain intact after 20 weeks of HFD feeding (39, 200). A potential instigating factor for adipocyte death in obesity is adipose tissue hypoxia, which can lead to either necrosis or apoptosis in various cell types and tissues (23, 117)

Adipose tissue oxygenation was shown to be reduced in the adipose tissue of obese C57BL/6 mice and this reduction was correlated with increased expression of hypoxia-inducible factors 1 α and 2 α (HIF-1 α and HIF-2 α) (173). Hypoxia caused increased expression of TNF- α , IL-1, and IL-6 (239), suppressed expression of the insulin-sensitizing adipokine adiponectin, and induced apoptosis and necrosis in the 3T3-L1 cell line. HIF-1 α appears to be a critical regulator of the adipose tissue dysfunction observed in obesity. A transgenic mouse with constitutively overexpressed HIF-1 α suffers from worsened glucose tolerance and insulin resistance which was associated with increased macrophage infiltration into the white adipose tissue (78). Furthermore, patients suffering from sleep apnea, a breathing disorder which results in intermittent periods of hypoxia, are predisposed to developing systemic insulin resistance (90, 196).

The generation of an immune response to self-antigen necessitates the abnormal presentation of antigen and an escape from the normal processes of peripheral tolerance. Although the process of apoptosis in the adipose tissue of obese mice could unmask adipocyte antigens, it is not clear why peripheral tolerance would be impaired in obesity. One factor which plays a key role in the maintenance of central and peripheral tolerance is B cell-activating factor (BAFF). Overexpression of BAFF has been observed in mouse models of SLE and SLE patients and has been implicated in the survival of autoreactive B cells. Interestingly, BAFF is secreted by adipocytes (4, 99) and the circulating levels of BAFF are increased in the context of obesity (80). The presence of BAFF, paired with increased apoptosis of adipocytes, could be sufficient to generate an autoreactive immune response to adipose tissue antigens.

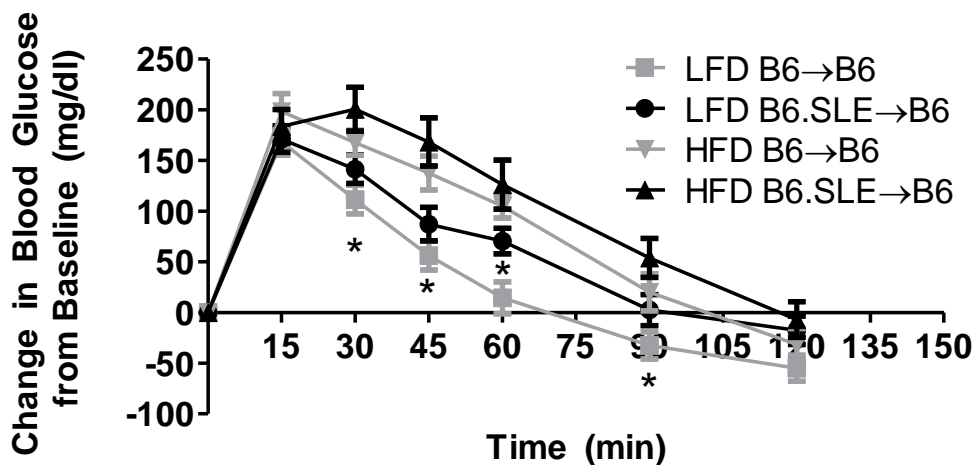


Figure 25. Glucose tolerance in B6.SLE radiochimeras. Six-week-old lethally irradiated B6 mice received a bone marrow transplant from a six-week-old B6 or B6.SLE donor. Recipient mice were rested for two weeks before being assigned to LFD or HFD groups. After 10 weeks of feeding, a glucose tolerance test was completed. After 5-6 hours of fasting, mice were injected with 1.25 g glucose per kg body weight and blood glucose was measured using a One Touch Ultra glucometer. n=9-10 mice/group. Data are expressed as change in blood glucose from baseline versus time. * $p < 0.05$ between LFD-fed B6.SLE and B6 mice via two-way ANOVA.

The experiments in Chapter 2 were not designed to assess the relative contributions of cellular immunity factors (e.g. a break in B cell or T cell tolerance to self-antigen) and tissue factors (e.g. abnormal exposure of self-antigen by adipocytes) to the development of glucose intolerance and adipose tissue insulin resistance in B6.SLE mice. However, a pilot study completed with B6.SLE radiochimeras shows that transplant of bone marrow from B6.SLE mice into B6 recipients is sufficient to cause glucose intolerance in recipient mice (Figure 25). In this experiment, bone marrow was harvested from 6 week-old male B6 or B6.SLE mice and was transferred into lethally-irradiated 6 week-old B6 mice. Previous studies by our laboratory showed that transfer of B6.SLE bone marrow was sufficient to induce autoimmunity and accelerated atherosclerosis in recipient mice (199). Transplanted mice were rested for 2 weeks randomly assigned to a LFD (10% Kcal from fat) or HFD (45% Kcal from fat) diet group. After

10 weeks of feeding, glucose tolerance was measured as described in Chapter 2. As seen in

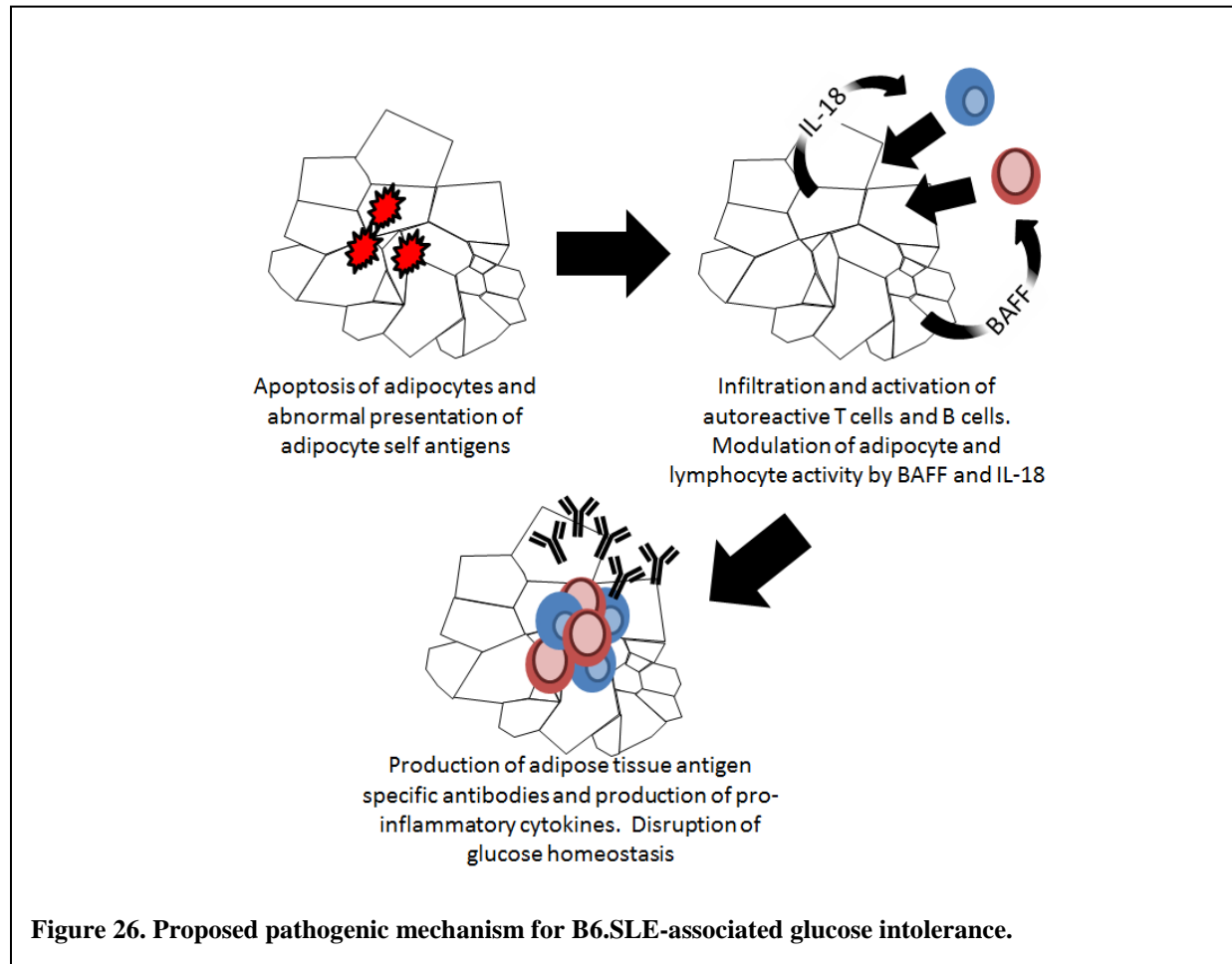


Figure 25, LFD-fed B6 mice that received B6.SLE bone marrow had significantly worsened but relatively mild glucose intolerance than those that had received B6 bone marrow. There was also a trend towards higher glucose intolerance in HFD-fed B6 mice. However, B6.SLE glucose tolerance in both diet groups was equal to or lower than B6 controls after 15 and 20 weeks of feeding (data not shown). This suggests that hematopoietic cells are sufficient to cause mild glucose intolerance after 10 weeks of feeding, but that lupus-related genetic susceptibility must be present in tissues in order to maintain worsened glucose tolerance. Since the contribution of tissue-specific factors was not assessed in this experiment, future experiments should also include B6.SLE recipients in order to address this question.

The collective results from our studies and from the work of other laboratories form the basis of the following proposed mechanism (Figure 26). Adipose tissue antigens become unmasked and are abnormally presented to the cells of the adaptive immune system in B6.SLE mice. Adipose antigen-reactive T and B cells escape the mechanisms of peripheral tolerance that normally prune autoreactive lymphocytes, and CD8⁺ T cells infiltrate the adipose tissue and modulate the activity of B cells to produce adipose tissue reactive IgG. These antibodies bind to adipocytes and cause damage through engagement of the adaptive and innate immune system. Finally, direct attack of adipocytes and the secretion of pro-inflammatory cytokines causes insulin resistance resulting in glucose intolerance.

Future Directions

Several components of the proposed model remain to be tested experimentally. The most important of these components is the contribution of IgG to the pathogenesis of adipose tissue insulin resistance and glucose intolerance. As noted above, Winer et al. showed that passive immunization with IgG from obese mice was sufficient to induce insulin resistance in recipient mice. In order to determine the contribution of IgG to glucose intolerance in the B6.SLE strain, we will harvest IgG from the serum of LFD-fed B6.SLE and B6 mice, and these antibodies will be used to passively immunize recipient B6 mice. Glucose tolerance tests and adipose tissue insulin signaling will be used to assess the metabolic function in the passively immunized mice. If passive immunization is found to cause glucose intolerance and insulin resistance, we will attempt to determine the target antigens of these antibodies. Emerging proteomic technology may facilitate the description of the putative adipose tissue targets of antibodies generated in the setting of SLE. One approach for antigen discovery would be “antigen panning,” in which adipose tissue lysates from LFD-fed B6.SLE mice would be applied to a 2D electrophoresis gel.

Antibody from obese and SLE-prone mice would be applied to the 2D blot and antibody-tagged proteins would be identified using mass spectrophotometry. Such an approach could identify adipose tissue antigen targets in B6.SLE-prone mice that may provide clues about the antigen targets of IgG in the setting of obesity.

It is also important to examine the role of BAFF in the pathogenesis of glucose intolerance and insulin resistance in the B6.SLE strain. BAFF is a cytokine that can promote the survival of autoreactive B cells and has been found to be elevated in the circulation of SLE patients and SLE-prone mice (35). Additionally, serum BAFF levels are associated with increased insulin resistance in obese mice and adipocytes have been shown to produce significant amounts of BAFF and to express the BAFF receptor (80). Therefore, BAFF could be a factor that links the break in tolerance of B cells to the disruption of glucose homeostasis in B6.SLE mice. Furthermore, it is possible that adipocytes produce the majority of BAFF in the setting of SLE. Serum BAFF levels were significantly increased in B6.SLE mice (Figure 12), but it is not clear whether this cytokine contributes to glucose intolerance. To address this question, we will inhibit BAFF activity through the use of neutralizing antibodies against BAFF and the BAFF receptor and will use recombinant BAFF to determine whether BAFF excess exacerbates metabolic disease in B6.SLE mice. Additionally, we will use conditional BAFF and BAFF receptor knockout mice to specifically ablate expression of BAFF and BAFF receptor in adipocytes to determine whether BAFF signaling is necessary to induce glucose intolerance and adipose tissue insulin resistance in the B6.SLE model.

An additional future investigational target is the cytokine IL-18. IL-18 is produced by macrophages and dendritic cells and affects the function of NK cells and T cells (208). IL-18 levels have been shown to be elevated in the settings of SLE and obesity (228, 231) and IL-18

levels positively correlate with metabolic syndrome in SLE patients (209). Like BAFF, IL-18 is a potential link between autoimmunity and glucose intolerance in the B6.SLE strain. Currently, it is not known whether IL-18 levels are increased in B6.SLE mice. It will be necessary to complete IL-18 ELISAs in order to determine whether levels are abnormal in B6.SLE mice. Furthermore, IL-18 neutralizing antibodies, recombinant IL-18 and conditional IL-18 knockouts can be used to determine whether this cytokine contributes to glucose intolerance in B6.SLE mice.

In conclusion, we described glucose intolerance and adipose tissue insulin resistance in the B6.SLE mouse model of SLE that was not associated with increased adiposity. Although these findings are novel in the B6.SLE strain, we were not able to determine the mechanistic basis of these metabolic changes. Several pieces of data, including increased proportions of IgG⁺ B cells and CD8⁺ T cells in the SVF of B6.SLE mice (Figures 9 and 10) and increased levels of serum BAFF in B6.SLE mice (Figure 12) offer tantalizing clues about the pathogenic mechanisms that disrupt normal glucose homeostasis in B6.SLE mice. The research directions described above could lead to the characterization of the factors that contribute to insulin resistance in the B6.SLE model and the SLE patient population.

SUMMARY AND CONCLUDING REMARKS

In this work we have shown for the first time that the B6.SLE mouse model of lupus is predisposed to the development of white adipose tissue insulin resistance and glucose intolerance. These impairments in glucose homeostasis are independent of body composition and occur in the setting of a diabetogenic B cell phenotype which is similar to that observed in diet-induced obesity models of insulin resistance. We showed that kidney disease in B6.SLE mice was characterized by glomerular tuft hypertrophy, increased infiltration by CD8⁺ T cells and increased renal tissue expression of pro-inflammatory cytokines in which was equally severe in LFD-fed and HFD-fed SLE mice.

Overall, this body of work shows that the B6.SLE strain is a suitable mouse model of the insulin resistance observed in the SLE patient population. Although HFD feeding did not exacerbate renal disease in B6.SLE mice, it is possible that other dietary factors such as vitamins could impact pathogenesis of the disease. The studies described in this dissertation form a strong foundation for future investigations into the mechanisms by which autoimmunity can affect metabolism and by which metabolism can impact the pathogenesis of autoimmune disease.

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