UNDERSTANDING THE PATHOGENESIS OF ACCELERATED ATHEROSCLEROSIS IN SYSTEMIC LUPUS ERYTHEMATOSUS: A ROLE FOR T CELL DYSREGULATION

By

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CHAPTER I

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune inflammatory disorder characterized by the production of autoantibodies to a variety of self-antigens, most notably double stranded DNA (dsDNA) and other nuclear antigens. The chronic inflammatory nature of SLE is hypothesized to lead to many co-morbidities including, but not limited to, renal disease, vasculitis, anemia, neuropathies and premature accelerated atherosclerosis. While the exact cause of SLE remains a mystery, there are a number of factors that are thought to contribute to disease pathogenesis including gender, race, environmental factors and genetics.

SLE disproportionately affects women, especially women of child bearing age. Although the exact etiology is unknown, it is thought that high estrogen levels, which are at their peak in this age range, contribute to the gender bias (1). Additionally, race is thought to be a major factor in SLE onset as African-American and Hispanic women are more likely to develop SLE compared to their Caucasian counterparts. With regard to the environment, studies have linked exposure to certain toxins, such as heavy metals and silica as well as exposure to ultraviolet light, to SLE disease. Genetic predisposition is also associated with SLE pathogenesis as polymorphisms and mutations in a number of genes including the Fcγ receptor, the complement receptor C1q, and tumor necrosis factor α (TNFα) have all been associated with SLE (1, 2).

In addition to the lack of understanding regarding the predisposing risk factors for SLE, the trigger for SLE onset is likewise unknown, and most likely multifactorial. According to recent studies, ineffective clearance of apoptotic cells may be a main culprit behind SLE. Under normal circumstances, apoptotic cells and debris are cleared through

a phagocytic process termed efferocytosis. However, in SLE, this apoptotic debris accumulates in tissues, allowing the immune cells to be exposed to self-antigens to which they otherwise would not be exposed. These defects in apoptosis trigger a chronic sterile inflammatory environment that is thought to lead to the loss of B cell tolerance. This in turn mediates the production of anti-nuclear antibodies, which can then form immune complexes. Immune complex deposition in tissues and organs exacerbates inflammation and leads to tissue damage typically seen in SLE patients. (3) Furthermore, these events result in the over-expression of pro-inflammatory cytokines, most notably type I interferons, which have been shown to mediate disease activity in both humans and mouse models (4-7). These events and others are thought to initiate and sustain SLE pathogenesis.

SLE and Cardiovascular Disease. Atherosclerosis, one of the most common cardiovascular diseases (CVD), continues to be a significant cause of morbidity and mortality despite recent advances in diagnosis and therapies. While it is widely recognized that hypertension, dyslipidemia and hypercholesterolemia predispose to atherosclerosis, studies in the past decades have revealed that the etiology of this disease is more complex than originally thought. Recent evidence suggests that the immune system is important in atherosclerosis pathogenesis and that these interactions occur early in the disease process (8).

Nearly thirty-five years ago, Urowitz *et al.* (9) first documented what was referred to as a bi-modal pattern of mortality in SLE, where early deaths in SLE were attributed to active SLE end organ disease, such as renal failure, while later deaths were mostly cardiovascular related. Since this pioneering discovery, many follow-up studies have demonstrated that, with all other risk factors being equal, the incidence of coronary artery disease in women with SLE is five to nine times higher compared to women without SLE. (10-12) Even more striking is the finding by Manzi *et al.* (13) that in

premenopausal women—an age group normally protected against CVD—having SLE increases the likelihood of suffering from myocardial infarction by 50 times compared to their non-SLE premenopausal counterparts. All of these studies indicate that both classical and non-classical risk factors play a pivotal role in SLE-accelerated atherosclerosis. However, the mechanisms of accelerated CVD in SLE remain to be elucidated.

In the following sections of this introduction we will: **1)** briefly summarize the association between autoimmunity and atherosclerosis; **2)** summarize recent data highlighting risk factors associated with atherosclerosis and SLE-accelerated atherosclerosis; **3)** highlight current models used to study these phenomena and **4)** state the goal(s) of the subsequent chapters.

Autoimmunity and Atherosclerosis. While the role of the immune system in atherosclerosis is fairly well established, it is not completely understood. Over the past two decades, the literature describing modulation of atherosclerosis by the immune system has continued to grow. In general, the body of work can be summarized by stating that the role of immunity in atherosclerosis is complex and, depending on the cell or immune axis of choice, can be either pro-atherogenic or anti-atherogenic. Therefore, it is probably not surprising that immune dysregulation would have detrimental effects on cardiovascular health. There is a growing body of evidence supporting a causal link between chronic autoimmune inflammation and development of accelerated atherosclerosis. Although much is still not known regarding autoimmunity and atherosclerosis, many studies have illustrated a correlation between several autoimmune diseases and CVD (12, 14, 15). To date, the best characterized autoimmune diseases associated with atherosclerosis include rheumatoid arthritis (RA), antiphospholipid syndrome and SLE.

RA and CVD. RA is characterized by inflammation, mainly of the synovial joints. Increased expression of adhesion molecules, matrix metalloproteinases and pro-inflammatory cytokines all contribute to bone and joint erosion in RA. These processes are hypothesized to contribute to accelerated atherosclerosis in patients with RA (15, 16). Furthermore, an accumulation of $CD4^+$ T cells within both the synovial fluid and atherosclerotic plaques point to a role for lymphocytes in propagating the atherosclerotic process (17). T cells from RA patients are unique in that they lack expression of the co-stimulatory molecule CD28. As a result, they do not depend on the B7/CD28 pathway for co-stimulation (15). This expanded T cell population has been associated with clinical markers of atherosclerosis (15, 18) and a study by Gerli *et al.* (17) found that RA patients had increased $CD4^+CD28^-$ T cells compared to control patients. This was accompanied by increased intima-to-media thickness and arterial endothelial dysfunction. This study and others indicate that modulating T cell response would be an attractive therapeutic target in RA-associated CVD.

APS and CVD. Antiphospholipid syndrome is an autoimmune disease characterized by excessive production of antibodies against phospholipids, mainly cardiolipin and β2-glycoprotein1 (β2GP1). This disease can cause dangerous blood clots due to increased formation of circulating immune complexes, and can lead to miscarriage and premature birth in pregnant women. Phospholipids play an integral role in cardiovascular disease and several studies have uncovered a link between APS and cardiovascular disease. In human studies, β2GP1 was found in the atherosclerotic plaque, mostly in association with CD4+ T cells (19). Immune complexes composed of antibodies against oxLDL/β2GP1 are capable of being taken up via Fcγ receptors and facilitating the differentiation of macrophages into foam cells (20). Moreover, studies have shown that anti-cardiolipin antibodies contribute to accelerated atherosclerosis by inducing endothelial activation and the adherence of monocytes to the endothelium (21). In

addition to occurring alone, APS can also be presented in conjunction with SLE. The remainder of this introduction will highlight features relevant to SLE and SLE-accelerated cardiovascular disease (SACVD).

Risk factors for SACVD. Several clinical studies have suggested that while traditional risk factors for cardiovascular disease—such as hypertension, dyslipidemia, and diabetes mellitus—can be present in the SLE population, these risk factors do not fully explain the increased prevalence of cardiovascular disease (9, 11, 22). Recent evidence suggests that a number of factors contribute to SACVD. Therefore, it is not surprising that since the association of premature CVD with SLE was discovered, basic and pre-clinical studies have been focused on determining the mechanism(s) driving this very serious co-morbidity in SLE patients.

While dyslipidemia is a well-known risk factor for atherosclerosis, clinical studies have demonstrated that abnormal lipoprotein functions may contribute to SACVD. HDL is known for its participation in cholesterol efflux. In addition to efflux, HDL can also regulate oxidation of LDL and inhibit adhesion molecule expression adding to its anti-atherogenic functions. However, under chronic inflammatory conditions such as SLE, normal HDL can lose its anti-oxidant capacity. This HDL is said to be more pro-inflammatory and therefore thought to have deleterious effects in both traditional CVD and SACVD (23). A study using autoimmune *gld* mice detected a significant reduction in HDL cholesterol and paraoxonase-1 activity, independent of HDL biogenesis. This phenotype was attributable to increased autoantibodies against apo-AI (24). Moreover, a clinical study observed that women with SLE have increased pro-inflammatory HDL, which was strongly associated with a 17-fold increased risk for CVD (25). Although not seen in this study, studies using other cohorts have found that SLE is also associated with an overall decrease in HDL and apoA-I levels and this decrease correlates with

increased SACVD risk. (26, 27) The similar phenotypes seen in mice and human SLE studies could perhaps hint at HDL dysregulation as a culprit in SACVD.

Mouse models of SLE and SACVD. Clinical studies have been extremely useful in determining predictors for SACVD risk. However, because SLE and atherosclerosis are complex diseases, human studies to elucidate causal mechanisms for autoimmune-accelerated CVD could prove difficult. As with many animal models for human disease, the availability of mouse models for SLE can be staggering. These models include mice that develop SLE spontaneously, drug induced models of SLE and gene-knockout animals, such as the FcγRIIB-deficient mouse, which develops lupus, but only on a C57Bl/6 background (28). To complicate the issue, many of the SLE mouse models only develop certain features of the human disease. For example, the MRL-*Fas*^{lpr} mouse is a good model for the cutaneous skin lesions often seen in human SLE patients (29). However, except for the fluorouracil-induced model, skin lesions are not common in the other lupus animals (30). In addition, few of the animal models develop the lupus-associated arthritis seen in human SLE. All of the animal models develop the characteristic anti-dsDNA antibodies and glomerulonephritis, although to varying degrees. (30, 31)

To date, there are relatively few mouse models specifically used to study the mechanism of SACVD (see Table 1). Published in 2004, the *gld.*apoE$^{-/-}$ mouse model, which contains an inactivating FasL mutation, was found to be more susceptible to atherosclerosis. Aprahamian *et al* (32) proposed that impaired macrophage function and inadequate clearance of apoptotic bodies were responsible for the observed accelerated atherosclerosis in their model. An alternative mouse model of SACVD was generated by Feng and colleagues using apoE$^{-/-}$ Fas$^{-/-}$ mice (33). The authors of this study observed that in addition to the presence of lupus-like disease and increased antibodies to

Table 1. Mouse Models of SLE-Accelerated Atherosclerosis

Animal Model	Diet	Increased Atherosclerosis	Renal disease	Cholesterol	Spleno-megaly	Antibody Production	Other Findings
gld.apoE$^{-/-}$ (32)	Western Diet (12 wks)	Yes	Yes	↓	Yes	Yes	• ↑ apoptosis • Impaired clearance of apoptotic debris
	Chow diet (12 wks)	Yes	Yes	↓	Yes	Yes	
LDLr.Sle1.2.3 (34, 35)	Western diet (8 wks) (34)	Yes	Yes	↓	Yes	Yes	• ↑ T cell activation • ↑ T cell accumulation in plaque
	Chow diet (8 wks) (35)	Yes	Yes	↓	Yes	Yes	
apoE$^{-/-}$Fas$^{-/-}$ (33)	Chow diet (5 mos.)	Yes	Yes	↓	Yes	Yes	• Ostopenia • Accumulation of apoptotic debris
MRL/*lpr*. apoE$^{-/-}$ (36)	Chow diet (24 wks)	Yes	n/a	↑	n/a	Yes	-
cGVHD induced lupus in apoE$^{-/-}$ mice (36)	Chow diet (24 wks)	Yes	n/a	↔	n/a	Yes	• ↓ marginal zone B cells

oxidized phospholipids, apoE$^{-/-}$ Fas$^{-/-}$ mice also uniquely develop osteopenia while exhibiting increased apoptosis similar to *gld.*apoE$^{-/-}$ mice. Correspondingly, Ma *et al.* (36) combined apoE$^{-/-}$ mice with three separate models of SLE and reported similar results.

While the abovementioned studies have significantly advanced our understanding of SACVD, one can argue that because SLE is likely a polygenic complex disease, it may be difficult to make human correlates from studies conducted in single-gene knockout animals. The development of the NZM2410-derived congenic B6.*Sle* mouse strains has made it feasible to examine SLE and atherosclerosis together on the susceptible C57Bl/6 background. Through linkage analyses, Morel *et al.* (37) identified three major genomic intervals linked to SLE susceptibility in the NZM2410 mouse strain. Using these three chromosomal intervals, termed *Sle1*, *Sle2* and *Sle3,* the investigators made a series of single, bi-, and triple congenic mice on the atherosclerosis susceptible C57Bl/6 background (38, 39). While having one or two intervals can lead to varying symptoms associated with SLE, all three intervals are necessary to display a fully penetrant SLE phenotype similar to the disease in humans.

Our laboratory took advantage of this mouse strain and developed an animal model of SLE-accelerated atherosclerosis and demonstrated that transfer of SLE susceptibility by bone marrow transplantation increases atherosclerosis in LDLr$^{-/-}$ mice (34). The increase in atherosclerosis was later determined to be independent of diet and was accompanied by a three-fold increase in CD4^{+} T cell burden within the atherosclerotic lesion (35) (see **Chapter 2**). CD4^{+} T cells from the SLE-susceptible mice also displayed higher expression of activation markers such as CD69 (34) and CD40L (35). Further studies from our laboratory revealed that transfer of *Sle3*-associated T cell dysregulation alone to LDLr$^{-/-}$ mice was not sufficient to affect atherosclerotic lesion area but leads to exacerbated humoral immune responses that are frequently associated with atherosclerosis in LDLr$^{-/-}$ mice (40) (see **Chapter 3**). This could mean that all three

genetic loci are necessary to induce accelerated atherosclerosis in our model. Also, it could suggest that B cell hyperactivity and dysregulation may influence the T cell phenotype in SACVD. The latter would be an intriguing model as more data from recent literature show that certain B cell subsets may be pro-atherogenic in the absence of autoimmunity (41, 42). Insights from these and other animal models will be extremely useful in delineating mechanisms of SLE and SACVD pathogenesis as well as targets for treatment of SACVD.

The Immune System in SACVD. *Cytokines in SACVD.* Traditional pro-inflammatory markers have been associated with SACVD risk; these include increased serum levels of pro-inflammatory cytokines such as IFN-γ, IL-6, TNFα, IL-10 and TGF-β (43-45). While IFN-γ and TNFα are largely known as pro-atherogenic cytokines in both humans and mouse models it is still unclear whether IL-6 has strictly anti-atherogenic effects. One study showed that treatment of mice with IL-6 exacerbates atherosclerosis (46). However, another study by Schieffer *et al*. (47) showed that 1 year old apoE$^{-/-}$IL-6$^{-/-}$ mice had increased plaque area, although the plaques contained less inflammatory cell infiltration, indicating that IL-6 may have multiple roles in disease pathogenesis.

The traditional anti-inflammatory cytokines, IL-10 and TGF-β, have been shown to be protective in the more traditional mouse models of CVD: the apoE$^{-/-}$ and LDLr$^{-/-}$ mice (48-50). However, both SLE patients and most animal models exhibit elevated serum levels of IL-10 and this cytokine is thought to mediate SLE pathogenesis (51, 52). Therefore, it is not known whether responses to IL-10 in the context of SLE might exacerbate the atherogenic process in these patients. Interestingly, we have shown that treatment of SLE-susceptible LDLr$^{-/-}$ mice with mycophenolate mofetil (MMF) (i.e. Cellcept®) leads to dramatic reductions in atherosclerotic plaque burden and significant decreases in circulating levels of IL-10 (53) (see **Chapter V**). Therefore, further

investigations regarding the role of IL-10 in SLE-accelerated atherosclerosis are certainly warranted.

TGF-β, another largely anti-atherogenic cytokine, likewise remains a mystery in the context of SLE. While it has been found that TGF-β is protective against atherosclerosis (48) and that TGF-β deletion, specifically in T cells, accelerates atherosclerosis (54, 55), it is not known how TGF-β may function in SLE. Several studies have reported decreased TGF-β expression in lymphocytes of SLE patients, and one study found that peripheral blood mononuclear cells from SLE patients were resistant to exogenous TGF-β stimulation(56, 57). A study by Jackson *et al*. (58) examined the efficiency of TGF-β activation in SLE patients with early atherosclerosis. They found an inverse correlation between TGF-β activation and LDL levels along with IMT scores, where SLE patients with higher IMT and LDL levels had decreased TGF-β activation. These trends were not found in control patients, suggesting that this phenomenon was specific to SACVD. While many of the current studies show a favorable link between pro-inflammatory cytokines and SACVD, future studies are warranted in order to fully assess their role in the disease process.

Immunomodulators in SACVD. There have been very few published clinical trials that have examined the effect of immunomodulatory agents on SACVD. Statins, while widely known for their cholesterol lowering capabilities, can also control inflammatory responses making it an attractive therapy for SACVD. There have been a number of trials attesting to the lipid lowering abilities of statins in patients with high risk of CVD. Furthermore, recent studies suggest that high dose statin therapy may halt or even reverse the atherosclerotic process in non-SLE patients (59). A review from our laboratory highlights clinical trials that have tested the potential benefits of these drugs in treating SACVD (60).Interestingly, although perhaps disappointingly, a complete analysis of data from the Lupus Atherosclerosis Prevention Study (LAPS) recently

revealed that while atorvastatin lowers cholesterol in SLE patients, it does not protect them from CVD (61).

Investigations in our laboratory and others have also been undertaken to assess the usefulness of currently marketed immuno-modulatory agents in treating SACVD in mouse models. Treatment of *gld.*apoE$^{-/-}$ mice with simvastatin led to a significant reduction in autoantibody production, lymphoproliferation, lupus nephritis and atherosclerotic lesion area, compared to *gld* and apoE$^{-/-}$ control mice (62). Despite the fact that this study suggests that immuno-modulatory statins could prove beneficial in treating both SLE and SACVD in mice, the same results have unfortunately not been found in other models (53, 61, 63).

To test the hypothesis that therapies targeted toward CVD and SLE could ameliorate atherosclerotic disease progress and osteopenia in their model, Woo and colleagues treated apoE$^{-/-}$ Fas$^{-/-}$ mice with a statin and/or apo-AI mimetic (63).(63). Perhaps counter-intuitively, combination therapy led to an increase in plaque size. However, this was associated with a beneficial remodeling of the plaque with decreased macrophage infiltration and increased smooth muscle content. This is a pivotal study as it suggests that while inhibition of atherosclerosis progression may be the current readout of success in our human studies of SACVD, the role of therapeutics on modifying plaque stability may be of equal or greater importance.

Our laboratory recently evaluated the effectiveness of atorvastatin and MMF treatment in ameliorating SACVD progression. We found that similar to human SLE trials, treatment of LDLr.*Sle1.2.3* mice with atorvastatin reduced cholesterol levels with no effect on atherosclerosis. However, MMF treatment had an athero-protective effect with decreased CD4^{+} T cell migration into the lesion(53). While this is expounded upon in the manuscript, the results from this study and other human studies assessing the benefit of statin therapy in SLE imply that dysregulated lymphocytic activity could be the

predominant driver in SACVD and that lipid-lowering by itself cannot resolve these perturbations. These studies and others, again, emphasize the complexities associated with SACVD.

The Role of B cells in SACVD. B cells are central to both SLE and atherosclerosis pathogenesis. The production of auto-antibodies is a hallmark feature of SLE and one of the main markers used to diagnose the disease. B cells can serve as antigen presenting cells, secrete cytokines which skew T helper cell responses, and modulate immune responses. While primarily known for their function in antibody production, B cells have been shown to have both antibody-dependent and independent functions in lupus. B cell deficiency or depletion in lupus-prone MRL/lpr mice was shown to inhibit disease progression while there was little change in lupus nephritis progression in mice with B cells that were unable to secrete antibodies (64). Additionally, when B cell deficient mice were infused with serum from mice with autoantibodies, little to no nephritis was observed thus, supporting the varied functions of B cells in SLE (65). In humans, B cells have been a long standing target in the race for therapeutic interventions. There have been several studies using antibodies toward B cells and B cell signaling mechanisms, most notable are the LUNAR and EXPLOROR trials which looked at the efficacy of rituximab, an antibody directed against CD20, in SLE patients with (LUNAR) or without (EXPLOROR) lupus nephritis (66). Despite favorable preliminary data, both trials failed to meet their target expectations. However, there are several ongoing trials targeting B cells, which may prove to be promising in the treatment of lupus. An exciting development occurred earlier this year when the Federal Drug Administration approved the first drug specifically for the treatment of SLE since 1955. Benlysta® (Belimumab) is a human neutralizing antibody targeted against B Lymphocyte Stimulator (BlyS). BlyS, also known as BAFF (B-cell activating factor), is important for B cell selection, survival and activation (67, 68). Clinical trials have reported an effective, albeit modest, reduction

in disease activity compared to patients given placebo. It will be interesting to see how this drug and other BlyS inhibitors that are currently being investigated stand up against SLE.

The story of B cells in atherosclerosis has recently taking a sharp turn. While originally thought to be anti-atherogenic, recent data suggest that the effects of B cells on atherosclerosis may depend on their subtype and the antibody subclass they produce. An early study by Major *et al*. revealed that transfer of B cell deficient μMT hematopoietic cells into $LDLr^{-/-}$ mice led to aggravated atherosclerosis (69). Moreover, transfer of splenic B cells from $apoE^{-/-}$ mice to splenectomized mice resulted in protection from atherosclerosis (70). Studies from the laboratory of Joe Witztum have indicated that while titers of antibodies against oxidized LDL (oxLDL) correlate with cardiovascular disease risk, immunization of atherosclerosis-susceptible mice with oxLDL and malondialdehyde (MDA)-LDL resulted in protection against atherosclerosis through an anti-inflammatory Th2 mechanism. Additionally, the authors showed that the atheroprotective effects of oxLDL and MDA-LDL are due to IL-5 mediated stimulation of B-1 B cells (71). It was also demonstrated that these B-1 B cells secreted natural IgM antibodies, including the T15/E06 idiotype, showing athero-protective effects by blocking oxLDL uptake through scavenger receptors (72) and controlling the immune response against apoptotic bodies containing oxidized phospholipids Unfortunately, like many aspects of the immune system in atherosclerosis, things are not always how they first appear and the role of B cells in this disease is no different. In fact, the most recent data seem to point to a pro-atherogenic role for B cells. Specifically, depletion of mature B cells using an anti-CD20 antibody resulted in reduced atherosclerosis, while transfer of B-2 but not B-1 cells resulted in aggravated atherosclerosis (41, 42). Taken together, these studies indicate that B cell subsets may have divergent effects on atherosclerosis pathogenesis.

The Role of T cells in SACVD. T cells have a major role in SLE initiation and development. Their importance is underscored in studies showing that T cell depletion ameliorates disease while lack of T cells inhibits SLE development (73-75). Furthermore, the T cell phenotype in SLE patients and mouse models is characteristically different than normal T cells. These cells display a spontaneous hyperactive phenotype where there is a low threshold for activation, permitting increased T-B cell cooperation, increased antibody production by B cells and increased cytokine secretion. Additionally, these cells are resistant to antibody induced cell death and have altered signaling mechanisms. (76-78)

Two subsets of T lymphocytes have recently garnered attention in both the SLE and atherosclerosis fields. The first type, termed regulatory T (Treg) cells, is a subset of suppressor T cells that control autoreactivity and maintain immunologic homeostasis. Tregs are characterized by the expression of CD25 and the transcription factor Foxp3. Impaired Treg function has long been associated with autoimmune disease development and progression. Their dysfunction and/or deficiency has been reported in human SLE and mouse models of SLE suggesting that Treg dysfunction may be one of the driving forces in SLE pathogenesis (79-82). Along the same lines, Tregs have been associated with protection from atherosclerosis. $Foxp3^{+}$ Tregs have been detected in the atherosclerotic lesions of both mice and humans (83). Ait-Oufella *et al*. (84) first showed that Treg deficiency in $LDLr^{-/-}$ mice leads to enhanced atherogenesis and that transfer of Tregs into Treg-poor $apoE^{-/-}$ mice attenuated atherosclerosis and reduced T cell accumulation within the lesions of the mice. An additional study showed that transfer of Tregs to $apoE^{-/-}$ hosts also led to decreased atherosclerotic plaque size (85).

Another T cell subset most recently implicated in the pathogenesis of SLE and atherosclerosis is the T helper 17 (Th17) cell. Th17 cells are regulatory $CD4^{+}$ T helper cells characterized by their ability to secrete large amounts of the pro-inflammatory

cytokine IL-17. Studies have found elevated levels of IL-17 in the sera of patients with SLE and an increased frequency of $IL17^+$ cells in the peripheral blood of SLE patients (86, 87). Changes in the IL-17 secreting cell population have been noted in other animal models of SLE such as the SNF_1 and BXD2, where it was shown that blockade of IL-17 signaling reduces germinal center formation and antibody production (88, 89). These studies all indicate a potential role for Th17 cells in autoimmune disease progression.

The role of Th17 cells in the pathogenesis of atherosclerosis is not well defined. Increased circulating IL-17 was found in patients with coronary artery disease, and the authors from this study concluded that IL-17 and IFN-γ produced by T cells infiltrating the coronary artery induce pro-inflammatory responses in vascular smooth muscle cells (90). Similar to this finding in patients, Pejnovic *et al.* (91) found that mice doubly deficient in both apoE and IL-18 have increased atherosclerosis and unstable plaques due to an increase in the Th17 cell population. Several studies have shown that blockade of IL-17A either through genetic deletion, retroviral inhibition or IL-17A neutralizing antibody, mediated a significant reduction in aortic lesion area and cellular infiltration (92, 93). Transfer of IL-17 receptor deficient bone marrow into $LDLr^{-/-}$ host caused a 46% decrease in lesion area along with a decrease in macrophage content in the lesion (94). However, a divergent study by Taleb *et al.* (95) found that loss of suppressor of cytokine signaling 3 (SOCS3) expression leads to an IL-17-dependent reduction in lesion size. Additionally, IL-17 administration in mice reduced vascular T cell infiltration and atherosclerosis development thus indicating an athero-protective role for Th17 cells and IL-17. To complicate the issue even more, a recent study by Cheng *et al*. (96) found that the host origin of the IL-17 neutralizing antibody used in mice can lead to differential effects on lesion area. Specifically, mice treated with an anti-IL-17 antibody derived from rat led to a decrease in lesion area, while neutralizing antibody generated in mouse had no significant effect on atherosclerosis despite reduced IL-17 serum

levels. The role of IL-17 in atherosclerosis has been extensively reviewed by Taleb *et al*. (97). Given the disparity in results from studies of this fascinating cytokine, it is obvious that additional studies are warranted to shed light on the role of IL-17 in the atherosclerotic process.

Summary and Statement of the Problem

This introduction highlighted data from recent studies from our laboratory and others investigating the role of immunity in SLE and atherosclerosis. While the link between autoimmunity and cardiovascular disease has been firmly established, more work needs to be done in order to fully understand the mechanisms of these co-morbidities. Commonalities between the two diseases are undeniable. As outlined in Figure 1, both processes deal with dysregulation, inflammation and ultimately lead to end organ disease. However, the complex nature of both of these diseases makes it hard to study them together. For instance, atherosclerosis is thought to be a Th1 mediated disease process; however studies show that under hypercholesterolemic conditions, and as atherosclerosis progresses, a Th2 dominant environment is observed (98, 99).

In the following chapters, we attempt to elucidate the mechanism of SACVD in our model. The studies outlined in chapters 3-5 are an extension from our work summarized in **Chapter 2** as we try to understand the role of T cell dysregulation in SLE-accelerated atherosclerosis. Through these studies, we provide evidence that in our mouse model, T cells play an indispensable role in mediating and sustaining SACVD, potentially through their hyper-migratory capacity; and that modulating their function could be the key to effective treatment of both diseases.

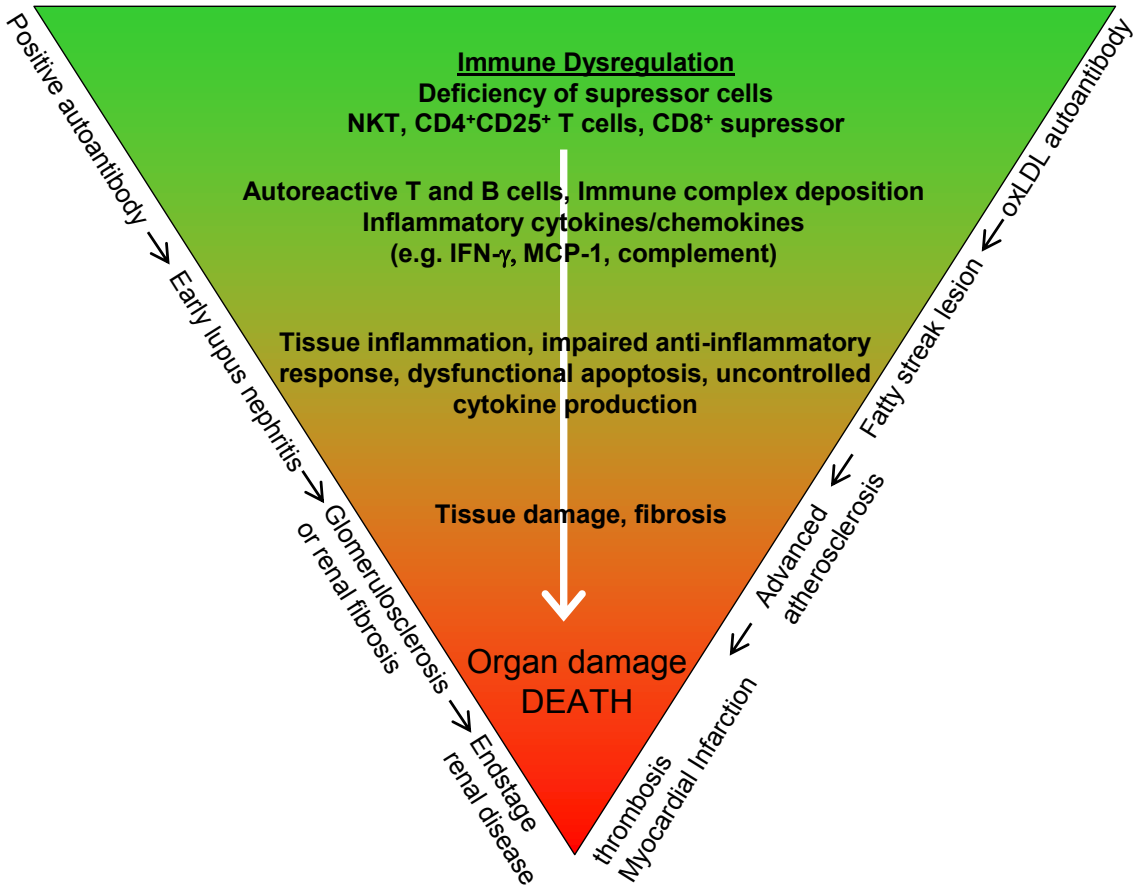

Figure 1. Immune mechanisms in common between SLE (left side of triangle) and atherosclerosis (right side of triangle). Both diseases involve immune dysregulation and increased inflammation leading ultimately to end organ disease.

CHAPTER II*

Accelerated atherosclerosis is independent of feeding high fat diet in systemic lupus erythematosus-susceptible LDLr$^{-/-}$ mice.

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease characterized by the production of a wide range of autoantibodies. Clinical complications because of SLE usually result in end-organ disease such as glomerulonephritis, arthritis, vasculitis and various neurological disorders(100). First recognized as a serious complication in SLE over 30 years ago, atherosclerosis has gained interest as a major cause of mortality in patients with lupus (100-103). In fact, with all other risk factors being equal, including hypertension, hyperlipidemia, diabetes and obesity, the risk of coronary events in patients with SLE is approximately eight times greater when compared with non-SLE controls and approximately 30% of deaths in SLE are atherosclerosis related (102, 103). Therefore, understanding how the presence of SLE exacerbates the atherosclerotic condition is essential to optimize risk reduction for cardiovascular disease (CVD) while treating the SLE-associated inflammation.

Atherosclerosis, like SLE, is a disease involving many cellular processes, and has classically been associated with hypercholesterolemia. A large body of evidence also supports inflammation and immunity in the pathogenesis of CVD. It is well known that macrophages and T cells are present in all stages of atherosclerotic lesions and promote inflammation by producing various cytokines, attracting smooth muscle cells

*Most of the data presented in this chapter were published in (35). Unpublished data presented here are denoted with an * preceding the paragraph.

and other lymphocytes and increasing plaque vulnerability (104). B-cell responses are also thought to be involved in the pathogenesis of atherosclerosis and for the most part are thought to be protective. Although the involvement of acquired immunity in atherosclerosis is strongly supported by these studies, mechanisms appear to be cell type dependent and multifaceted.

A recent study by our laboratory described the development of an animal model for accelerated atherosclerosis in the face of SLE. We made LDLr$^{-/-}$ mice susceptible to SLE by transferring hematopoietic cells from the congenic B6.*Sle1.2.3* mouse strain. This unique animal model of human SLE was developed by placing three lupus-susceptibility gene intervals identified in NZM2410 mouse strain on the C57Bl/6 background (37). Using this approach, we showed that making LDLr$^{-/-}$ mice susceptible to SLE increased atherosclerosis in the aortic root and increased inflammatory cell accumulation in lesions. However, in general, patients with SLE do not suffer from the severe hypercholesterolemia observed in LDLr$^{-/-}$ mice fed a high fat diet (e.g., cholesterol levels >500 mg/dL). Therefore, the current study was conducted to show that exacerbation of atherosclerosis in lupus-susceptible mice occurs under conditions of more moderate dyslipidemia as that observed in LDLr$^{-/-}$ mice on a normal chow diet (total cholesterol of approximately 200 mg/dL) and that overt accumulation of atherogenic lipoproteins (i.e., VLDL and LDL) can enhance SLE disease.

Materials and Methods

Mice. All mice used in these studies have been backcrossed onto the C57Bl/6 background. C57Bl/6 and LDLr$^{-/-}$ mice were originally obtained from The Jackson Laboratory and are maintained in our colony. The SLE congenic B6.*Sle1.2.3* strain has been described and characterized extensively (37-39, 105-108). The B6.*Sle1.2.3* mice

are essentially 97% genetically homologous to the C57Bl/6 strain with the NZM2410-derived lupus susceptibility loci accounting for approximately 3% of the genome. All mice are maintained in microisolator cages and used according to the guidelines and the approval of the Vanderbilt University Institutional Animal Care and Use Committee.

Production of radiation chimeras. Transfer of the wild type or lupus-susceptible bone marrow has been previously described (34).

Atherosclerosis studies. LDLr-deficient animals received either C57Bl/6 or B6.*Sle1.2.3* bone marrow. Sixteen weeks following transplantation, one half of the animals in each group were started on a high fat Western diet (21% milk fat and 0.15% cholesterol) for 8 weeks. The remaining mice were kept on chow diet for the same period of time. At the end of this time, animals were sacrificed and analyzed for the extent of atherosclerosis and the presence and severity of symptoms of SLE.

Immunohistochemistry. Staining for macrophages (Moma-2) and $CD4^+$ T cells was performed as previously described (34). CD11c staining for dendritic cells was conducted using a rat anti-CD11c primary antibody (BD Biosciences, San Jose, CA USA) followed by incubation with Texas red-conjugated anti-rat IgG (Vector Labs, Burlingame, CA, USA). Cells were visualized by fluorescent microscopy and quantified by counting the number of positive cells in lesions.

ELISAs. Serum titers for antibodies against oxLDL and dsDNA were conducted as previously described (34). ELISAs for antibodies against β2-glycoprotein I (β2GP1) was performed by coating a 96-well Maxisorb plate with 10 μg/mL of purified β2GP1 in PBS. Plates were blocked and mouse serum was added at a dilution between 1:1000 and 1:5000 and incubated overnight at 4 °C. Plates were washed with 0.5% Tween/PBS and incubated with HRP conjugated goat anti-mouse IgG (Promega, Madison, WI, USA) for 1 h at RT. Reactions were developed using the TMB substrate (BD Biosciences).

Serum lipoprotein analyses. Total serum cholesterol and triglyceride were measured in fasted mice using a colorometric assay as previously described (34). Fast performance liquid chromatography (FPLC) was conducted by separating lipoproteins on a Superose 6 column (Amersham Promega, Piscataway, NJ, USA) followed by cholesterol measurement in each fraction as described (34, 69).

Measurement of systolic blood pressure. Systolic blood pressure was measured using a tail cuff BP-2000 instrument (Visitech Systems, Apex, NC, USA) on conscious, preconditioned mice as described (34).

Purification and activation of $CD4^+$ T cells. $CD4^+$ T cells from the spleens of C57Bl/6 and B6.*Sle1.2.3* congenic mice were isolated by positive selection using magnetic beads conjugated to anti-CD4 antibodies according to the manufacture's protocol (Miltenyi Biotec, Auburn, CA,USA). Cells were then stimulated with Phorbol myristate acid (PMA) (10ng/ml) and ionomycin (1µg/mL) for 2 h at 37 °C and 5% CO_2. Cells were then washed, stained with anti-CD40L antibody (BD Biosciences) and analyzed by flow cytometry unless otherwise indicated.

***Western blotting.** $CD4^+$ T cells were isolated and stimulated as referenced above. Cells were then harvested and nuclear and cytoplasmic fractions prepared using the Thermo Scientific NE-PER kit. Nuclear and cytoplasmic fractions were loaded on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked in 3% milk/PBS-T, and incubated with the primary antibody for one hour. This was followed by incubation with an anti-mouse IgG-HRP (Promega, Madison, WI) secondary antibody for 45 minutes. Primary antibodies: mouse anti-NFATc1 or mouse anti-NFATc2 (both from BD Pharmingen).

Statistical analyses. Statistical analyses were conducted using PRISM 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). For data showing a normal distribution, significant differences were calculated using an ANOVA with a Bonferroni post-test. For

data that require a nonparametric test, a Kruskal–Wallis test was conducted with a Dunns post-test.

Results

High fat Western diet increases dsDNA antibody titers and increases mortality in lupus-susceptible LDLr$^{-/-}$ mice.

Lethally irradiated LDLr$^{-/-}$ mice received either C57Bl/6 or lupus-susceptible B6.*Sle1.2.3* congenic bone marrow. Recipient mice are hereafter referred to as LDLr.B6 and LDLr.*Sle1.2.3*, respectively. Sixteen weeks following transplantation, mice were either fed a high fat Western diet for eight additional weeks or left on normal rodent chow. LDLr.*Sle1.2.3* mice fed with high fat Western diet had a 37% mortality rate, whereas LDLr.*Sle1.2.3* mice on a chow diet only showed a 10% mortality rate (Figure 2A). Control LDLr.B6 mice tolerated both diets with a 100% survival rate at the end of the 24-week study. At the time of sacrifice, the LDLr.*Sle1.2.3* mice had significantly higher titers of anti–double stranded DNA (anti-dsDNA) serum antibodies compared with LDLr.B6 controls (Figure 2B). This was evident independent of diet. In addition, the LDLr.*Sle1.2.3* mice on Western diet had significantly increased anti-dsDNA antibodies compared with chow fed LDLr.*Sle1.2.3* mice. Body weight at the time of sacrifice did not differ between the LDLr.B6 and LDLr.*Sle1.2.3* mice in either diet group (Figure 2C). The data confirm that the SLE phenotype was transferred to the LDLr-deficient mice. In addition, they show that feeding lupus-susceptible mice a high fat Western diet increases mortality and disease severity as determined by dsDNA autoantibody titer.

Increased immunoglobulin deposition in kidneys correlated to the dsDNA antibody titer in that LDLr.B6 mice on chow diet had the least staining and LDLr.*Sle1.2.3* mice on Western diet had the most staining (Figure 3A). Similarly, urine protein was the greatest in LDLr.*Sle1.2.3* mice on Western diet (Figure 3B). Finally, systolic blood

pressure in LDLr.*Sle1.2.3* mice on a Western diet was significantly greater than LDLr.*Sle1.2.3* mice on chow or LDLr.B6 mice on either Western or chow diet (Figure 3C). Collectively, these data suggest that renal function was decreased in LDLr.*Sle1.2.3* mice on high fat diet.

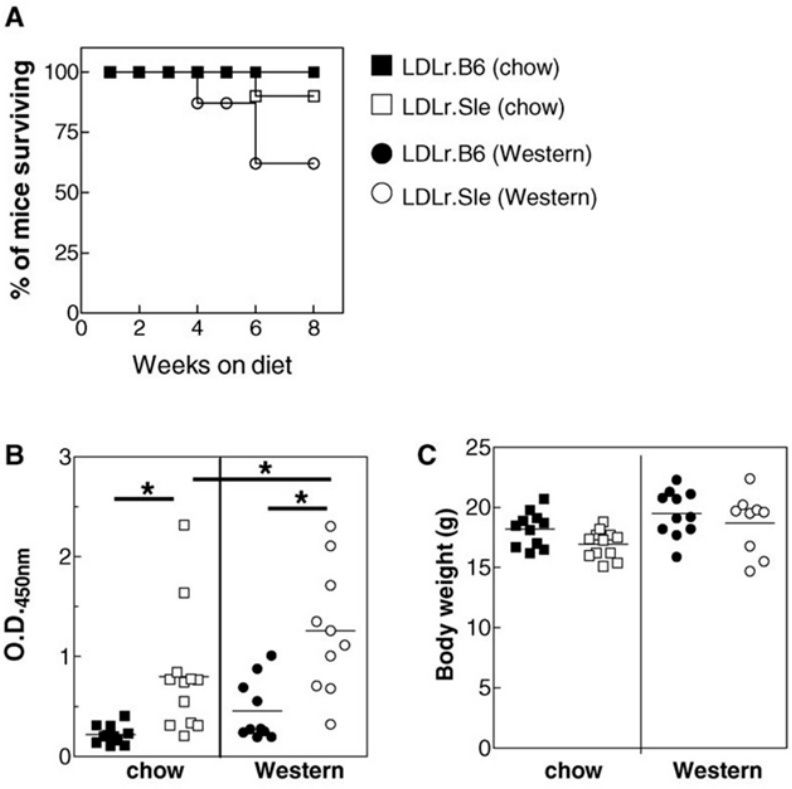

Figure 2. Severe dyslipidemia increases mortality and serum titers of dsDNA antibodies in LDLr.*Sle1.2.3* mice. (A) Percentage of mice (total of 9–13 mice per group) surviving following feeding chow diet (squares) or Western diet (circles) for 8 weeks. **(B)** Serum titers for anti-dsDNA antibodies in LDLr.B6 (solid symbols) and LDLr.Sle (open symbols) mice fed a chow or Western diet. **(C)** Body weights for LDLr.B6 (solid symbols) and LDLr.Sle (open symbols) mice fed a chow or Western diet. *$p < 0.05$ as determined by one-way ANOVA. Data represent two identical experiments.

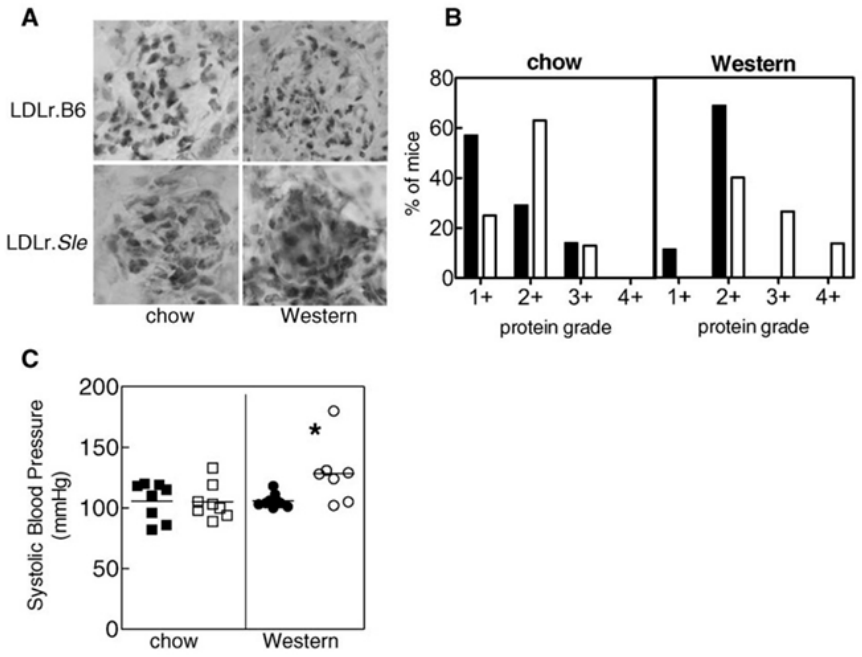

Figure 3. Immune complex deposition, urine protein and blood pressure are increased in LDLr.*Sle1.2.3* mice fed high fat diet. (A) LDLr.Sle mice fed high fat diet exhibit increased immune complex deposition in glomeruli as detected by immunohistochemistry. Shown is one representative mouse per group. Kidney sections from a total of five mice per group were analyzed with similar results. **(B)** Urine protein grade in mice (3–7 mice per group) was determined by Chemstix at the time of sacrifice. LDLr.Sle mice (open bars) have increase urine protein compared with LDLr.B6 controls (closed bars). **(C)** LDLr.Sle mice (open circles) on Western diet have increased systolic blood pressure compared with LDLr.Sle mice on chow diet (open squares) and LDLr.B6 mice fed chow (closed squares) or Western diet (closed circles). *$p < 0.05$ as determined by one-way ANOVA. Data represent two identical experiments.

Table 1

Serum cholesterol and triglyceride

Group	N	Cholesterol mg/dL (±SEM)	Triglyceride mg/dL (±SEM)
Chow diet			
LDLr.B6	11	230.4 (11.3)	76.6 (13.0)
LDLr.*Sle1.2.3*	12	239.4 (10.7)	86.2 (12.5)
Western diet			
LDLr.B6	10	971.4 (57.4)	311.6 (10.6)
LDLr.*Sle1.2.3*	10	767.1 (73.3)[a]	201.8 (26.6)[a]

[a] $P < 0.05$ compared with LDLr.B6 mice on Western diet.

Lupus-susceptibility increases atherosclerosis in LDLr-deficient mice in the absence of overt dyslipidemia.

Our previous study showed that transfer of lupus susceptibility increases atherosclerosis in LDLr$^{-/-}$ mice on a Western diet (34). However, feeding high fat diet in this animal model results in extreme elevations in circulating cholesterol; often greater than 1,000 mg/dL. This level of cholesterol would not be considered physiological to the SLE patient. We hypothesized that SLE could exacerbate atherosclerosis under more physiologic levels of serum lipoproteins (i.e., approximately 200 mg/dL). Measurement of atherosclerotic lesions in the proximal aorta showed that LDLr.*Sle1.2.3* mice had increased atherosclerosis compared with LDLr.B6 controls independent of diet (Figure 4A). In fact, the increase in atherosclerosis in LDLr.*Sle1.2.3* mice on chow compared with controls was actually greater (approximately 2.0-fold) than the difference between the mice fed Western type diet (approximately 1.3-fold). Analysis of the cellular composition of plaques by immunohistochemistry showed similar macrophage (Moma-2) and dendritic cell (CD11c) content among all groups of mice (data not shown). However, CD4$^+$ T-cell content was increased approximately threefold in the LDLr.*Sle1.2.3* mice compared with LDLr.B6 animals (Figure 4B). This increase was independent of diet because both chow and Western diet fed animals showed similar percentages of CD4$^+$ T cells in lesions. Stimulation with PMA and ionomycin showed an increase in CD40L expression in B6.*Sle1.2.3* primary CD4$^+$ T cells compared with wild type C57Bl/6 mice (Figure 4C).

The increase in atherosclerosis in LDLr.*Sle1.2.3* mice fed a chow diet occurred in the absence of changes in serum cholesterol or triglyceride levels (Table 2) or cholesterol-containing lipoprotein distribution as determined by FPLC (Figure 5, left panel). Mice fed a Western diet showed increased serum cholesterol and triglycerides compared with chow fed mice (Table 2). In addition, as reported in our previous study

(34), serum cholesterol and triglycerides were slightly, but significantly, decreased in LDLr.*Sle1.2.3* mice. The difference in total cholesterol was associated with decreased non–high density lipoprotein (HDL) cholesterol (Figure 5, right panel). These data show that transfer of SLE to LDLr$^{-/-}$ mice can increase atherosclerosis independent of large increases in total serum cholesterol or triglycerides associated with high fat diet feeding. In addition, they show that increased numbers of CD4^{+} T cells in the lesions of LDLr.*Sle1.2.3* mice is also independent of severe hyperlipidemia.

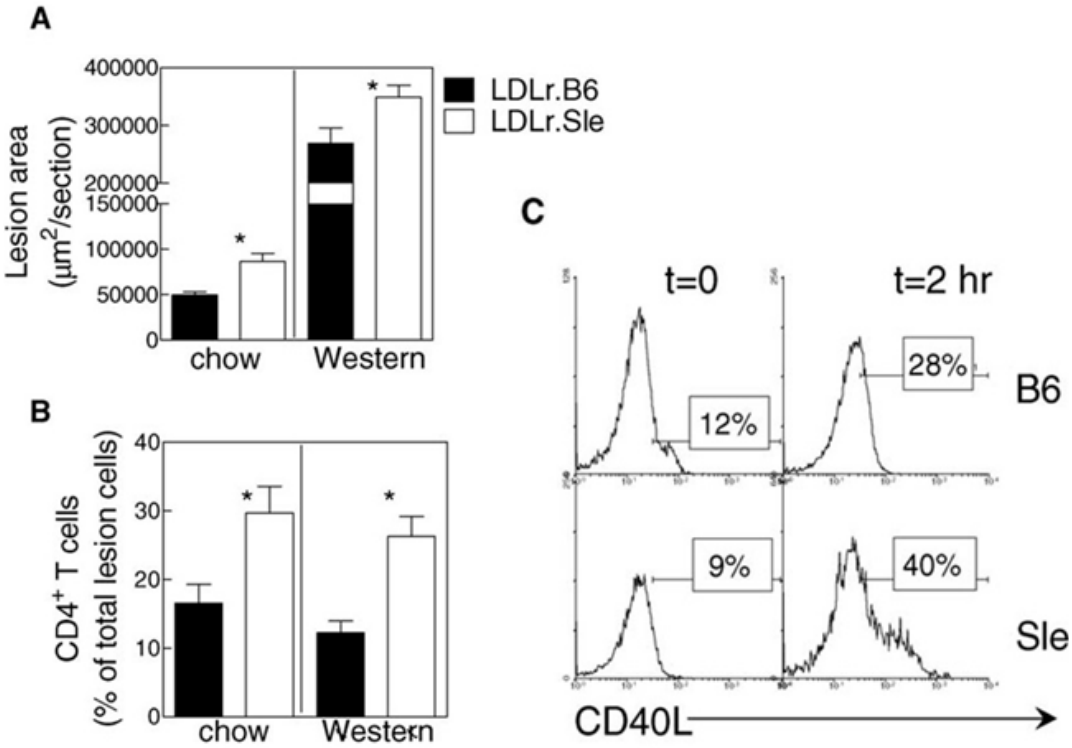

Figure 4. Transfer of lupus-susceptibility to LDLr-deficient mice increases atherosclerosis independent of diet. (A) Average lesion area as determined by oil-red-O staining in LDLr.B6 (closed bars) and LDLr.*Sle* (open bars) mice. Lesion quantitation was performed on 9–11 mice in each group. Shown is data from two identical experiments. **(B)** Detection of CD4^{+} T cells in lesions of LDLr.B6 (closed bars) and LDLr.*Sle* (open bars) on chow or Western diet. Positive cells are expressed as a percent of all lesion cells as determined by DAPI staining. **(C)** Representative histograms of CD40L expression by stimulated T cells.Primary CD4^{+} T cells were isolated from C57Bl/6 (B6) and B6.*Sle*1.2.3 (Sle) mice and stimulated for 2 h with PMA and ionomycin. Shown is one of three experiments with similar results.

Lupus susceptibility increases oxLDL and phospholipid antibodies in LDLr-deficient mice.

To examine the effect of diet on production of immunoglobulin against modified LDL and phospholipid, we measured titers of oxLDL- and β2-glycoprotein I (β2GP1)–specific antibodies in sera of control and SLE-susceptible LDLr$^{-/-}$ mice. Table 3 contains the anti-oxLDL total antibody and IgG isotype titers from chow and Western diet fed mice. In general, the LDLr.*Sle1.2.3* mice had higher total antibody, IgG_1 and IgG_{2a} titers in serum compared with LDLr.B6 mice. This increase in oxLDL-specific IgG was independent of diet feeding (Table 3). In addition, chow fed LDLr.*Sle1.2.3* mice had significantly higher anti-oxLDL IgM levels compared with LDLr.B6 mice. The LDLr.*Sle1.2.3* mice fed a Western diet had increased, but not significantly higher, levels of anti-oxLDL IgM.

To determine whether the antibody response to oxLDL was associated with a Th2 or a Th1 T helper phenotype, we calculated the IgG_1 (Th2) to IgG_{2a} (Th1) isotype ratio. Independent of diet, the LDLr.*Sle1.2.3* mice had an increased IgG_1/IgG_{2a} ratio indicating that the immune response of these animals was skewed toward a Th2 phenotype. Additionally, the LDLr.B6 mice fed a Western diet appeared to have more of a Th1 type phenotype than LDLr.B6 mice on chow.

Antibodies to the phospholipid β2GP1 are present in patients with SLE and the anti-phospholipid syndrome (109, 110). These antibodies are thought to be associated with increased risk of CVD. Therefore, we determined the serum titer of β2GP1 antibodies in our chow and Western diet fed animals. LDLr.*Sle1.2.3* mice fed with chow diet had significantly higher total antibody, IgM and IgG_1 specific for β2GP1 compared with chow fed LDLr.B6 mice (Table 4). IgG_{2a} showed a trend toward increased levels in LDLr.*Sle1.2.3* mice, but did not reach statistical significance. In mice fed with Western diet, β2GP1-specific IgG_1 and IgG_{2a} were significantly increased in LDLr.*Sle1.2.3* mice

compared with control LDLr.B6 mice. Analysis of the IgG_1/IgG_{2a} ratio showed similar trends as seen with the oxLDL antibody titers. In general, the LDLr.*Sle1.2.3* mice had more of a Th2 phenotype compared with LDLr.B6 mice, independent of the diet fed. Additionally, LDLr.B6 mice fed a high fat diet appeared to have more of a Th1 phenotype. Collectively, these data indicate that there are quantitative and qualitative differences in the immune response to vascular disease–associated antigens between the LDLr.B6 and LDLr.*Sle1.2.3* mice.

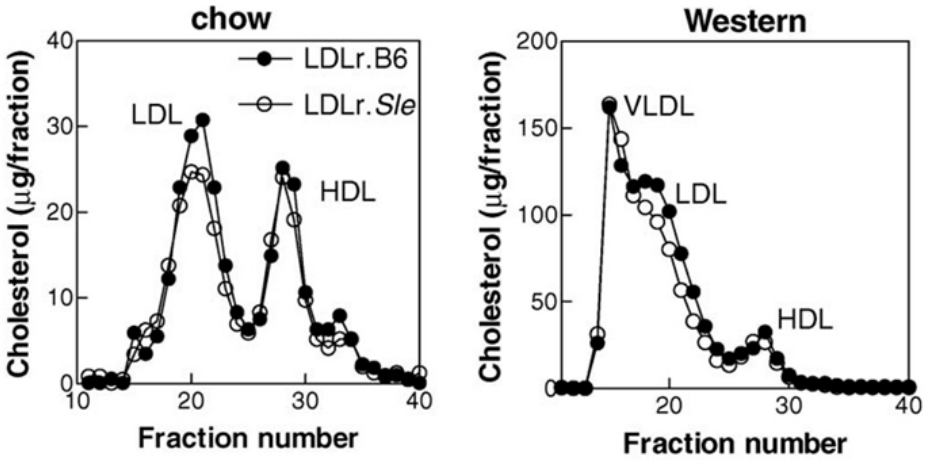

Figure 5. FPLC analyses of serum cholesterol lipoprotein distribution. Lipoproteins were separated by size-exclusion chromatography and assayed for cholesterol as described in 'Materials and Methods'. Serum from LDLr.B6 (closed circles) and LDLr.*Sle* (open circles) was pooled before undergoing separation and analysis (9–11 mice per group). Similar profiles were obtained from individual mice.

Table 3

Serum titres of oxLDL-specific antibodies

Group[a]	Total Ig (±SEM)[b]	IgM (±SEM)	IgG_1 (±SEM)	IgG_{2a} (±SEM)	IgG_1/IgG_{2a} (±SEM)
Chow					
LDLr.B6	0.402 (0.104)	0.770 (0.114)	0.302 (0.093)	0.431 (0.097)	0.661 (0.020)
LDLr.*Sle1.2.3*	0.900 (0.128)[c]	1.267 (0.102)[c]	0.956 (0.095)[c]	0.743 (0.069)[c]	1.307 (0.101)[c]
Western					
LDLr.B6	0.398 (0.108)	1.01 (0.180)	0.236 (0.090)	0.527 (0.131)	0.399 (0.059)[c]
LDLr.*Sle1.2.3*	0.839 (0.090)[c]	1.335 (0.069)	1.058 (0.024)[c]	0.944 (0.006)[c]	1.119 (0.022)[d]

[a] n = 6-9 mice per group

[b] average OD_{450nm} ± SEM.

[c] $p<0.05$ compared to LDLr.B6 mice on the same diet as determined by one way analysis of variance.

[d] $p<0.05$ compared to LDLr.B6 mice on chow diet as determined by one way analysis of variance.

Table 4

Serum titres of β2-GPI-specific antibodies

Group[a]	Total (±SEM)[b]	IgM (±SEM)	IgG_1 (±SEM)	IgG_{2a} (±SEM)	IgG_1/IgG_{2a} (±SEM)
Chow					
LDLr.B6	0.149 (0.033)	0.120 (0.030)	0.128 (0.061)	0.158 (0.065)	0.981 (0.187)
LDLr.*Sle*	0.690 (0.180)[c]	0.543 (0.172)[c]	0.588 (0.115)[c]	0.370 (0.122)	2.040 (0.304)[c]
Western					
LDLr.B6	0.467 (0.186)[d]	0.253 (0.101)	0.069 (0.024)	0.179 (0.063)	0.474 (0.084)
LDLr.*Sle*	0.514 (0.140)	0.402 (0.089)	0.722 (0.051)[c]	0.635 (0.093)[c]	1.270 (0.219)[c]

[a] n = 6-8 mice per group.

[b] average OD_{450nm} ± SEM.

[c] $p<0.05$ compared to LDLr.B6 mice on the same diet as determined by one way analysis of variance.

[d] $p<0.05$ compared to LDLr.B6 mice on chow diet as determined by one way analysis of variance.

*** T cells from B6.Sle mice display dysregulated phenotypes similar to those seen in SLE patients.**

T cells are central to the immune system and abnormalities associated with this cell type can lead to a number of diseases. Evidence from a number of studies has shown that T cells from SLE patients harbor significant signaling defects which impact their function and correlate with disease severity (76-78). Given our results of increased T cell hyperactivation and infiltration into the lesion, we analyzed T cells from B6.*Sle1.2.3* mice to determine if they harbored similar T cell defects as observed in SLE patients.

Th17 cells –named for their ability to secrete large amounts of IL-17—are a highly pro-inflammatory T cell subset and an imbalance between regulatory T cells (Tregs) and Th17 cells is thought to drive the progression of many inflammatory diseases including SLE and atherosclerosis (111-115). In SLE, the Treg compartment of both patients and mouse models is known to be impaired. Conventional $CD4^+CD25^+Foxp3^+$ Tregs were found to be decreased and/or dysfunctional in humans and mice, including our model (116-119). Induced Tregs (iTregs), which are induced in the periphery from naïve $CD4^+CD25^-$ T cells by IL-2 and transforming growth factor β (TGF-β) administration (120-122), are another Treg subset known to be important for maintaining immunologic tolerance. While these regulatory T cell populations have been investigated in other disease models, their status in our SLE model was unknown. These studies compelled our laboratory to examine these regulatory populations in B6.*Sle1.2.3* mice. Our analyses revealed that B6.*Sle1.2.3* mice displayed decreased TGF-β-induced Tregs compared to B6 mice (Figure 6B). Conversely, B6.*Sle1.2.3* mice had a two-fold increase in $CD4^+$IL-17^+ T cells, which was similar to other mouse models of autoimmune disease (Figure 6A).These data provide evidence that regulatory T cell populations are

imbalanced in our model and that this imbalance could partly facilitate the accelerated atherosclerosis in the LDLr.*Sle1.2.3* mice.

Given the similarities between our mouse model and observations in SLE patients, we decided to further characterize the T cell defect in B6.*Sle1.2.3* mice**.** We first examined Nuclear Factor of Activated T-cells (NFAT) expression. NFAT is a calcium dependent transcription factor whose translocation to the nucleus leads to the transcription of target genes involved in T cell responses, including IL-2 and CD40L (77). Studies in SLE patients have shown that there is increased nuclear accumulation of NFAT, in the absence of its transcriptional co-activator, activator protein-1, which is associated with increased CD40L expression and decreased IL-2 expression (123, 124). In our laboratory, we isolated $CD4^+$ T cells from control B6 mice and B6.*Sle1.2.3* mice with active disease and stimulated these T cells *in vitro* with PMA and ionomycin. We found that after stimulation, the nuclear fraction from B6.*Sle1.2.3* T cells contained increased NFATc1 and NFATc2 nuclear protein expression compared to B6 mice (Figure 7A). Along with increased nuclear NFAT expression, we also observed increased CD40L expression (Figure 4C) and decreased IL-2 levels (Figure 7B). These results illustrate that T cells from B6.*Sle1.2.3* possess similar signaling defects as observed in SLE patients, making it an even more relevant model to study the pathogenesis of SLE accelerated atherosclerosis.

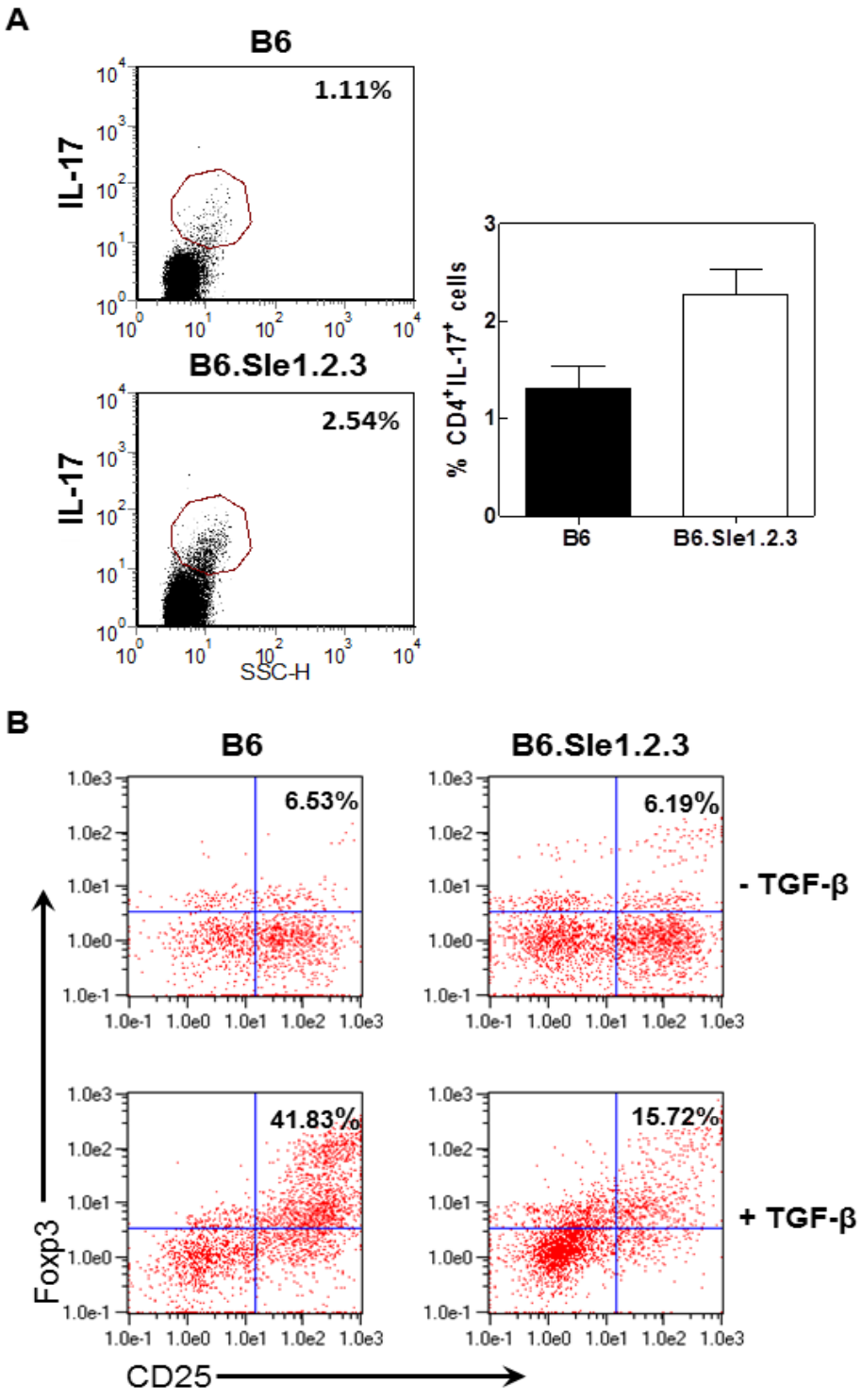

Figure 6. iTreg differentiation is abrogated in B6.*Sle1.2.3* mice while Th17 cells are increased. **(A)** Representative FACS plot of Tregs. $CD4^+CD25^-$ splenocytes were stimulated with plate bound α-CD3 and α-CD28 for 72 hours in the presence or absence of TGF-β (2ng/ml). Plots are gated on $CD4^+$ cells. In the left panel, the *p*-value is less that 0.05 compared to B6 mice. (n=3-4 mice per group). **(B)** Representative FACS plot of Th17 cells. Gated on $TCR\beta^+CD4^+$ cells. One of three repeated experiments.

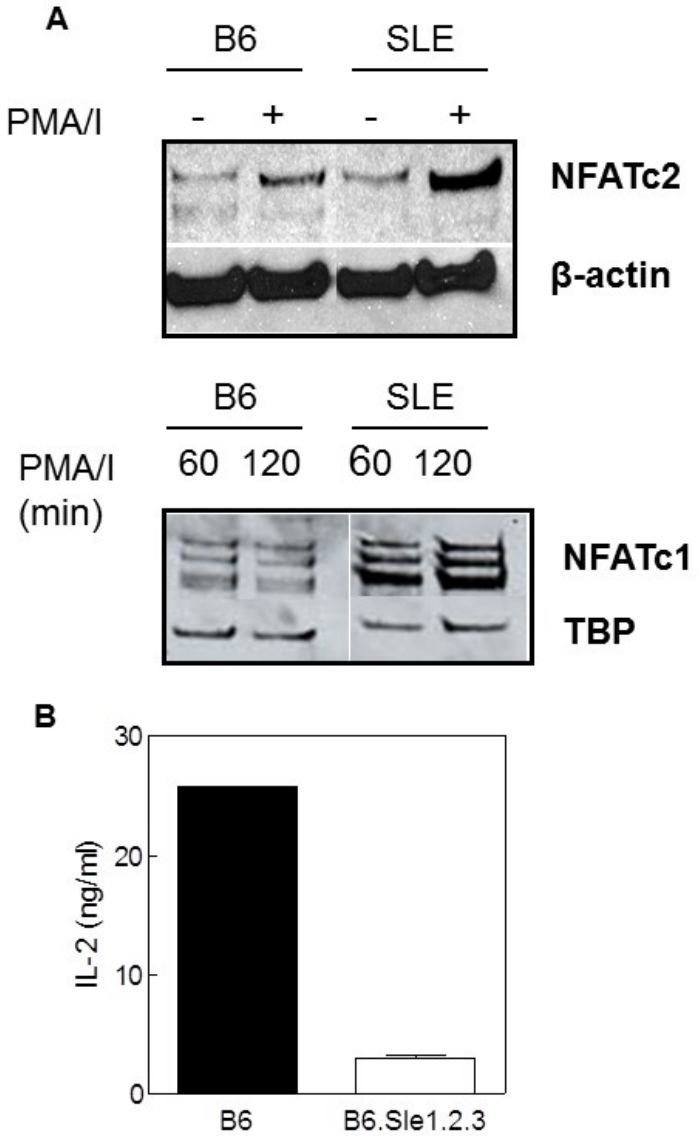

Figure 7. B6.*Sle1.2.3* T cells have decreased nuclear NFAT expression and decreased IL-2 secretion upon stimulation. (A) Representative blot of nuclear NFATc2 (top panel) and NFATc1 (bottom panel) expression in $CD4^+$ T from B6 and B6.*Sle1.2.3* (SLE) mice. Splenocytes were harvested from mice and $CD4^+$ cells isolated by magnetic separation. Isolated cells were cultured with PMA and ionomycin. NFAT, β-actin and TATA binding protein (TBP) expression was detected by Western blot. One of at least three repeated experiments. **(B)** IL-2 secretion by $CD4^+$ T cells stimulated for 48 hours with PMA and ionomycin. Closed bars represent B6 while open bars represent B6.*Sle1.2.3* mice. $N \geq 3$ mice per group. Experiment was repeated at least twice with similar results.

Discussion

Individuals suffering with SLE are at increased risk for developing accelerated forms of atherosclerosis and vascular disease. Because many patients with SLE do not fall into the traditional risk group for atherosclerosis, the underlying etiology for its acceleration remains largely unknown. Until recently, SLE and atherosclerosis studies have been hampered by the lack of an appropriate animal model that simultaneously develops both diseases.However, recent studies by our laboratory (34) and others (32, 62, 125) have reported that transfer of the SLE phenotype to atherosclerosis-susceptible mouse strains (e.g., $LDLr^{-/-}$ or $apoE^{-/-}$ mice) results in dysregulated immunity, chronic inflammation and increased atherosclerotic lesions. However, we also observed that when placed on the traditional atherosclerosis-inducing Western diet, the LDLr.*Sle1.2.3* animals exhibited increased mortality compared with the LDLr.B6 controls with the first animals dying as early as 2 weeks following diet initiation. Mortality by the end of the 8-week feeding period was approximately 40%. Whether this increased death was due to renal failure, heart failure or both is currently unknown. However, in the surviving LDLr.*Sle1.2.3* mice, urine protein was elevated indicating the presence of some degree of kidney disease.

In general, patients with SLE do not develop the severe dyslipidemia observed in $LDLr^{-/-}$ mice fed with Western diet (cholesterol ≥600 mg/dL). In addition, increased serum cholesterol is not predictive of accelerated atherosclerosis in patients with SLE.(126, 127) Therefore, we decided to test the hypothesis that the transfer of SLE is sufficient to exacerbate atherosclerosis in the presence of more physiological levels of serum cholesterol (approximately 200 mg/dL). To test this hypothesis, we transferred lupus-susceptible or resistant bone marrow to $LDLr^{-/-}$ mice and fed the mice either chow or high fat Western diet. In line with our previous study, we observed a 37% mortality rate

at the end of 8 weeks in LDLr.*Sle1.2.3* mice fed with Western diet compared with LDLr.*Sle1.2.3* mice fed with chow diet or LDLr.B6 mice fed either diet (Figure 2A). In addition, LDLr.*Sle1.2.3* mice fed a high fat diet had higher serum titers of dsDNA antibodies compared with LDLr.B6 mice on high fat and LDLr.*Sle1.2.3* mice on chow diet. These data are in line with previous work reporting that lupus-susceptible NZB/W F1 mice fed a high fat diet develop increased anti-dsDNA and cardiolipin antibody titers (128, 129), increased MHC class II expression on accessory cells (130), increased cytokine production and more severe lupus nephritis (131). However, the dietary effects in these studies were observed over a period of 2–9 months of feeding. In the current study, the LDLr.*Sle1.2.3* mice were only fed diet for 2 weeks when the animals started dying. In two separate studies, Lin *et al*. (129, 130) reported a decreased life span in the NZB/W F1 mice fed with high fat diet. The average life span of these animals was 285 days compared with the low fat fed controls, which lived for an average of 389 days. Because we were interested in measuring atherosclerosis, we did not allow the mice to proceed past 8 weeks of diet feeding. However, by 6 weeks of diet feeding, the LDLr.*Sle1.2.3* mice on high fat diet had already suffered significant mortality compared with the other three groups indicating that the life span of these animals was greatly decreased. Because the LDLr.*Sle1.2.3* mice fed a high fat diet had dsDNA titers even greater than the chow fed LDLr.*Sle1.2.3* mice, we were not surprised to see that the immunoglobulin deposition, urine protein grade and systolic blood pressure were also elevated in these animals (Figure 3). Although most individuals with SLE do not exhibit total serum cholesterol levels greater than 200 mg/dL, the fact that the LDLr.*Sle1.2.3* mice fed high fat diet have even greater disease symptoms compared with the LDLr.*Sle1.2.3* mice on chow diet suggests that severe dyslipidemia can further exacerbate SLE disease in these animals. Certainly, these data are consistent with the

clinical evidence showing that elevated serum total cholesterol in patients with SLE is associated with increased kidney pathology and death (132).

Examination of atherosclerosis in the proximal aorta of the mice showed that increased atherosclerosis in LDLr.*Sle1.2.3* mice compared with control LDLr.B6 controls was independent of high fat diet feeding (Figure 4). In fact, the LDLr.*Sle1.2.3* mice fed chow diet showed an even greater increase over controls compared with their high fat fed counterparts. Additionally, the average serum total cholesterol and triglyceride levels did not differ between the LDLr.B6 and LDLr.*Sle1.2.3* mice on chow diet and the serum lipoprotein distribution of cholesterol was similar (Figure 5). However, the LDLr.*Sle1.2.3* mice fed a high fat diet had decreased serum cholesterol and triglyceride with decreased non-HDL cholesterol. The accumulation of $CD4^+$ T cells in the atherosclerotic lesions of LDLr.*Sle1.2.3* was similarly not dependent of high fat diet feeding and $CD4^+$ T cells isolated from chow fed B6.*Sle1.2.3* mice show increased CD40L expression upon stimulation with PMA and ionomycin compared with C57Bl/6 controls. Collectively, these data suggest that the autoimmune dysregulation and perhaps chronic inflammation have a greater influence on atherosclerosis than serum cholesterol; a more traditional risk factor for atherosclerosis. More importantly, the data show that non-physiologically high serum cholesterol levels are not necessary to exacerbate atherosclerosis in the setting of lupus.

Although the lipoprotein distribution of cholesterol did not differ between $LDLr^{-/-}$ mice receiving either normal or lupus-susceptible bone marrow, we cannot exclude the possibility that the LDLr.*Sle1.2.3* mice harbor dysfunctional HDL. HDL functions not only in reverse cholesterol transport but also acts to prevent oxidation of LDL (133). When inflammation becomes chronic, HDL appears to lose its capacity to prevent the formation of oxLDL. In SLE, it has been shown that HDL function is abnormal in 45% of patients compared with only 4% of control (23). Therefore, in the SLE mice, it will be interesting

to determine whether we see similar decreases in HDL's anti-oxidative abilities compared with normal control animals.

Patients with SLE are known to develop autoantibodies to many atherosclerosis-associated antigens, such as oxLDL, β2-glycoprotein I, cardiolipin (134) and antibodies to atheroprotective proteins such as the HDL-associated apolipoprotein AI (135). A recent study by Svenungsson, *et al*. (136) reported a strong correlation between plasma concentrations of oxLDL and anti-oxLDL antibodies in SLE patients with coronary heart disease complications. The authors suggest using atherosclerosis-associated antibodies as a screen for identifying SLE patients with increased risk for the development of atherosclerosis. However, whether the autoantibody production in these studies was the cause of enhanced atherosclerosis or a secondary effect of other immune responses remains to be determined. In the current study, we observed an increase in the anti-oxLDL and β2GP1 serum IgG levels in LDLr.*Sle1.2.3* mice compared with control LDLr.B6 animals. This increase appeared to be diet independent as the LDLr.*Sle1.2.3* mice fed a Western diet did not show increased levels of antibodies compared with LDLr.*Sle1.2.3* mice fed with chow diet. Interestingly, the LDLr.*Sle1.2.3* mice show an IgG_1 bias for both anti-oxLDL and anti-β2GP1 antibodies compared with LDLr.B6 mice independent of diet. This suggests that high fat feeding in LDLr.*Sle1.2.3* mice skews the immune response toward a Th2-like phenotype. It has been shown in apoE-deficient mice that atherosclerotic lesion progression is accompanied by an increase in the oxLDL IgG_1 serum titers (98) and a bias toward Th2 immune responses. Therefore, these data support the hypothesis that the atherosclerotic disease in the LDLr.*Sle1.2.3* mice is more advanced that those seen in the LDLr.B6 controls.

Although titers of oxLDL antibodies are shown to correlate directly with severity of disease and are often used as markers of CHD risk (137-140), their role in the initiation and/or progression of atherosclerosis is not yet conclusive. In addition, it has

been shown that in patients with SLE, there is extensive antibody cross-reactivity between antiphospholipid (e.g., β2GP1) and oxLDL. Therefore, in the current study, it cannot be concluded that the oxLDL antibodies are unique from the β2GP1 antibodies. However, a recent report by Kobayashi *et al*. (141), suggested that anti-β2GP1 IgG significantly increases oxLDL/β2GP1 complex binding to macrophages and uptake via Fcγ receptors and, thus, may contribute to the atherosclerotic process. Therefore, one may hypothesize that the increase in β2GP1 antibody titers seen in the LDLr.*Sle1.2.3* mice accelerates uptake of modified atherogenic lipoproteins such as oxLDL/β2GP1 complexes and potentiates foam cell formation in these animals. Interestingly, although increased anti-β2GP1 antibodies are associated with increased risk of atherosclerosis, one study in mice showed that β2GP1 reactive T cells, and not antibodies, may be pathogenic (142). The authors reported that adoptive transfer of whole splenocytes, but not T-depleted splenocytes, from mice immunized with β2GP1 increased atherosclerosis in $LDLr^{-/-}$ animals in the absence of detectable antigen-specific antibody. These data would argue that antibodies against β2GP1 are a useful marker for cardiovascular risk but are not pathological. However, the current studies do not directly address either of these possibilities and ongoing studies in our laboratory are aimed at examining these hypotheses.

*We also examined regulatory T cell populations in B6.*Sle1.2.3* mice. The finding of increased Th17 and decreased Tregs was not surprising given data from other models of autoimmunity (88, 143, 144). *Ex vivo* expanded iTregs were found to delay or prevent autoimmune disease in mice. Su *et al.* (145) demonstrated that both freshly isolated and *ex vivo* expanded $CD4^+CD25^+$ T cells inhibited dsDNA antibody production in an autoimmune model of chronic graft versus host disease. Furthermore, Scalapino *et al.* (146) showed that transfer of iTregs delayed the onset of glomerulonephritis in the

NZB/W mouse model of lupus. These and other studies implicate a therapeutic potential for iTregs in modulating SLE disease progression.

Interestingly, it has been shown that induced Tregs are resistant to Th17 conversion by IL-6 (both *in vitro* and *in* vivo) and are able to inhibit autoantibody production in models of chronic graft-versus-host disease (147). In atherosclerosis, it is well known that Tregs are anti-atherogenic. However, these studies focus only on naturally occurring Tregs. While one study proposed an anti-atherogenic role for antigen specific regulatory T cells (148), to date there are no studies that exclusively examine the effect of iTregs on cardiovascular disease development and progression. Although this chapter does not directly address the role of iTregs and Th17 cells in SACVD, it is possible that the creation of methods to control the differentiation of these cell types may be advantageous. The "Addendum" will attempt to shed light on the differentiation and regulation of these two cell types as well.

In conclusion, in our current study, we have reported that severe dyslipidemia, as that seen in LDLr$^{-/-}$ mice fed a Western diet, can exacerbate the SLE phenotype and accelerate mortality beyond that previously reported in the NZB/W F1 mice fed with high fat diets (130). This suggests that perhaps the lipoprotein profile (i.e., increased VLDL and LDL cholesterol) can adversely affect the SLE disease process. Additionally, we also provide strong evidence that the accelerated atherosclerosis observed in LDLr.*Sle1.2.3* mice is more directly associated with immune hyperactivity and not due to increased non-HDL cholesterol or a secondary effect of autoimmune-mediated renal pathology associated with SLE. Planned future studies will allow us to delineate which specific immune cell type and function facilitates accelerated atherogenesis, ultimately leading to the identification of novel therapeutics that target both SLE and CVD.

CHAPTER III

The lupus susceptibility locus *Sle3* is not sufficient to accelerate atherosclerosis in lupus susceptible LDLr$^{-/-}$ mice.

Introduction

Our laboratory currently uses a triple congenic mouse model as a model of SACVD in order to elucidate the immune mechanism(s) of atherosclerosis in the setting of SLE. As discussed in **Chapter 2**, this model is derived from three chromosomal intervals linked to SLE genetic susceptibility. When SLE susceptible hematopoietic cells are transferred to atherosclerosis susceptible LDLr$^{-/-}$ mice, they develop accelerated atherosclerosis. This increase in atherosclerosis is independent of diet and accompanied by a three-fold increase in CD4^{+} T cell burden within the atherosclerotic lesion area (refer to **Chapter 2**) (34, 35).

The laboratories of Drs. Laurence Morel and Edward Wakeland are using this B6.*Sle1.2.3* model and their derivative single and bi-congenic models to dissect and determine the genes responsible for SLE-susceptibility. Our laboratory is taking a similar approach in regards to SACVD as the minimal genetic interval in B6.*Sle1.2.3* mice necessary to accelerate atherosclerosis is unknown. Data from our LDLr.*Sle1.2.3* mice suggest that T cell hyperactivity and lesional T cell accumulation drive the atherosclerotic process in SACVD. Genetic studies have determined that the susceptibility locus *Sle3* is associated with increased CD4:CD8 ratios, increased T cell activation and decreased activation induced cell death (108, 149, 150). Additionally, it has been shown that many of these phenotypes are due to *Sle3* expression on antigen presenting cells (108).These data led us to hypothesize that T cell dysregulation

mediated by the lupus susceptibility interval *Sle3* can facilitate the accelerated atherosclerosis seen in mice susceptible to SLE. In the current study, we examined whether *Sle3* expression on hematopoietic cells is sufficient to accelerate atherosclerosis in LDLr$^{-/-}$ mice.

Materials and Methods

Mice. C57Bl/6J (B6) and B6.129S7-Ldlrtm1Her/J (LDLr$^{-/-}$) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the Vanderbilt University animal care facility. All procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee. The B6.NZMc7 (B6.*Sle3*) mice, a generous gift from Edward Wakeland at the University of Texas Southwestern Medical Center, are C57Bl/6 mice congenic for the NZM2410-derived chromosome 7 lupus susceptibility interval. This single congenic strain has been described previously (108, 149, 150).

Production of radiation chimeras and atherosclerosis studies. Transfer of bone marrow was accomplished by bone marrow transplantation as described previously (34). Female LDLr$^{-/-}$ mice received either C57Bl/6 or B6.*Sle3* bone marrow, hereafter referred to as LDLr.B6 and LDLr.*Sle3*, respectively. Sixteen weeks after transplantation, mice were placed on a high-fat Western diet (21% milk fat, 0.15% cholesterol) for eight weeks. Mice were then sacrificed and analyzed for the degree of atherosclerosis and the presence and severity of symptoms associated with SLE.

Serum Lipoprotein Analysis. Total serum cholesterol and triglyceride were measured in mice fasted for at least four hours using a colorimetric assay as described previously (151). Lipoprotein distribution was determined by using FPLC.

Serum Cytokine Analysis. Serum cytokine levels were analyzed using the Milliplex Mouse Cytokine/Chemokine kit according to manufacturer's protocol and detected using Luminex® xMAP® (Millipore, Billerica, MA).

Enzyme-Linked Immunosorbent Assays (ELISAs). Serum titers of dsDNA were measured according to the method of Shivakumar *et al.* (152). Anti-oxLDL antibodies were measured as previously described (69). Serum antibody titers against β2-glycoprotein I (β2GP1) were measured by coating a 96-well Maxisorb plate with 10 μg/ml of purified β2GP1 in 1% bovine serum albumin (1% BSA)/PBS overnight. Plates were blocked in 1% BSA/PBS for two hours at room temperature. Mouse serum was added at a dilution between 1:500 and 1:5000 and incubated overnight at 4°C. Plates were washed with 0.5% Tween-20/PBS (PBS-T) and incubated with biotin-conjugated goat anti-mouse Ig(H+L) (SouthernBiotech, Birmingham, AL) for 45 minutes at room temperature then incubated with avidin-peroxidase for 30 minutes at room temperature. Plates were then washed with PBS-T and developed using TMB substrate (BD Bioscience). Anti-β2GP1 immunoglobulin isotype ELISAs were performed as described above using a biotin-conjugated goat anti-mouse IgG1, IgG2C or IgM (SouthernBiotech) secondary antibody. Cardiolipin antibody titers were determined by coating a 96-well Maxisorb plate with cardiolipin (Sigma-Aldrich) (15μg/ml in 95% ethanol). The cardiolipin ELISA was then conducted as described above for β2GP1. Serum was diluted 1:1000, 1:500 and 1:5000 for oxLDL, β2GP1 and cardiolipin ELISAs, respectively.

Immunohistochemistry. Staining for macrophages (MOMA-2) and $CD4^+$ T cells was performed as described previously (34, 35). Cells were visualized and staining quantified using Image-Pro Plus software (Media Cybernetics, Bethesda, MD).

Flow Cytometry. For flow cytometric analyses, spleens were removed and processed through a 0.70 μm mesh screen. Cells were counted, resuspended in 4% fetal bovine serum (FBS) in PBS with 0.5% sodium azide, and incubated with appropriate antibodies

for 40 minutes at 4°C. Cells were then washed and analyzed using a 5-Laser BD LSRII flow cytometer (BD Bioscence) and FacsDiva software (BD Bioscience). The following antibodies were used: TCRβ (H57–597), CD8α (53–6.7), CD4 (GK1.5), CD69 (H1.2F3), B220 (RA3-6B2), NK1.1 (PK136), CD44 (IM7), CD40L (MR1), CD11b (M1/70), CD40 (1C10), CD80 (16-10A1) and CD86 (GL1) (all purchased from BD Bioscience).

Statistical Analyses. Statistical analyses were conducted using PRISM 5.0 software (GraphPad Software Inc., La Jolla, CA). For data with a normal Gaussian distribution, a Student's t-test was used to calculate significant differences between groups. For data not normally distributed, a Mann-Whitney test was performed. A p value of < 0.05 was considered significant.

Results

Characterization of the lupus phenotype in LDLr.*Sle3* mice.

B6.*Sle3* mice are known to produce antibodies against dsDNA (39). To confirm the hematopoietic transfer of the lupus phenotype into LDLr$^{-/-}$ hosts, dsDNA antibody titers were measured in serum collected at time of sacrifice. As expected, LDLr.*Sle3* mice had higher dsDNA antibody titers compared to control (Figure 8A). Examination of spleen weights at the time of sacrifice showed that LDLr.*Sle3* mice had increased spleen:body weight ratios compared to LDLr.B6 mice (Figure 8B).

The congenic B6.*Sle3* mouse model is additionally associated with T cell hyperactivity, increased CD4/CD8 T cell ratios, and hyperstimulatory antigen presenting cells in the absence of spleen size differences when compared to B6 controls (108, 149, 150). We sought to determine if these differences in splenic cell populations could also be observed when the NZM2410-derived *Sle3* congenic interval is transferred hematopoietically to atherosclerosis susceptible LDLr$^{-/-}$ mice fed a Western diet. Spleen

cells were incubated with the panel of fluorophore-conjugated antibodies as outlined in the “Materials and Methods” section and cell populations were analyzed by flow cytometry. Flow cytometric analyses showed no significant differences in B cell, $CD8^+$ T cell, macrophage, dendritic cell or NK cell numbers in the spleens of LDLr.B6 and LDLr.*Sle3* mice (Figure 8C). However, we did observe a variable 2-fold increase in $CD4^+$ T cell numbers and CD4:CD8 T cell ratios. The increase in $CD4^+$ T cells was also accompanied by an increase in the activation marker CD40L and a trend toward increased percentages of $CD4^+CD69^+$ and $CD4^+CD44^+$ T cells in LDLr.*Sle3* mice (Figure 8D-E).

Transfer of lupus susceptibility interval *Sle3* is not sufficient to accelerate atherosclerosis in LDLr$^{-/-}$ mice.

Sixteen weeks after transplantation, mice were placed on a Western diet containing 21% milk fat and 0.15% cholesterol for eight weeks. After eight weeks, mice were sacrificed and analyzed for severity of atherosclerosis. Examination of atherosclerotic lesion area in the proximal aorta of LDLr.B6 and LDLr.*Sle3* by oil-red-O staining revealed no statistically significant difference in lesion area between the two groups (Figure 9A). In addition, the cellular composition of the atherosclerotic plaque, as assessed by Moma-2 and CD4 staining, was similar in both lupus-susceptible and control animals (Figure 9B and C).

Interestingly, as observed in our previous studies with LDLr.*Sle1.2.3* mice, measurement of serum cholesterol and triglyceride levels revealed a significant decrease in cholesterol in LDLr.*Sle3* mice compared to LDLr.B6 mice (Figure 10A). In addition, although not statistically significant, there was a trend for lower serum triglyceride levels in LDLr.*Sle3* mice compared to controls (Figure 10B). FPLC analysis revealed that the 20% decrease in serum cholesterol in LDLr.*Sle3* mice was due to a

decrease in the very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol fractions (Figure 10C). Overall, these results show that while there was no difference in lesion area and cellular composition of plaques between the two groups, the LDLr.*Sle3* mice did have lower cholesterol and triglyceride levels than control animals.

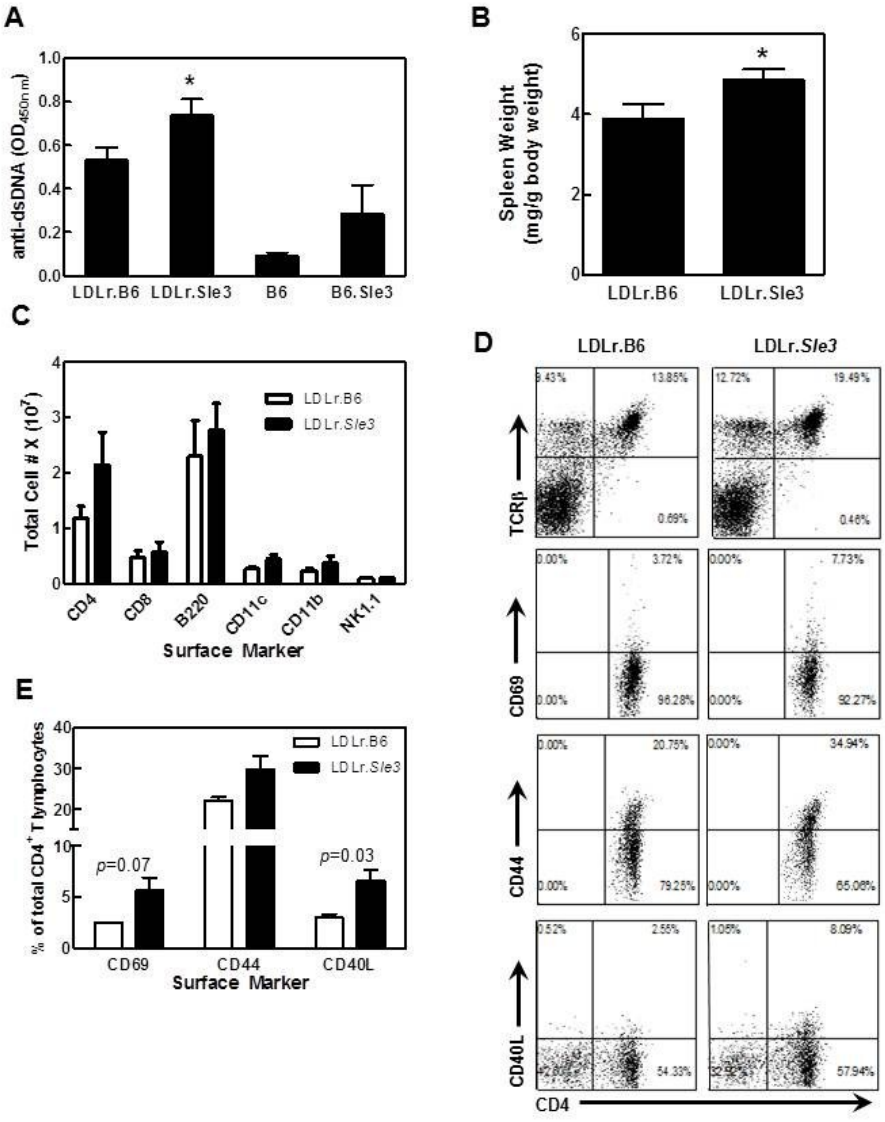

Figure 8. Characterization of lupus phenotype in LDLr.*Sle3* mice. **(A)** Serum titers of anti-dsDNA antibodies in LDLr.B6 (n=19) and LDLr.*Sle3* (n=17) mice. (1:1000 dilution; p=0.038). **(B)** Spleen:body weight ratio of LDLr.B6 (n=19) and LDLr.*Sle3* (n=17) mice (p=0.044). **(C)** Absolute numbers of spleen cell populations in LDLr.B6 (open bars) and LDLr.*Sle3* (closed bars) mice (n=3-6 mice per group). **(D)** Representative FACS plot of designated T cell activation markers in LDLr.B6 and LDLr.*Sle3* mice. **(E)** Percentage of $CD4^+$ T lymphocytes expressing the designated activation marker in LDLr.B6 and LDLr.*Sle3* (n=3 mice per group). * indicates a p value < 0.05 compared to LDLr.B6 mice.

Atherosclerosis associated antibody production is augmented in LDLr.*Sle3* mice.

We examined total immunoglobulin levels against the anti-phospholipid antibody cofactor β2GP1 and the phospholipid cardiolipin. Measurements of antibodies against both antigens are indicative of anti-phospholipid antibody titers. We observed a trend toward increased anti-β2GP1 (Figure 11A) and anti-cardiolipin (Figure11B) immunoglobulin levels in LDLr.*Sle3* mice although neither reached statistical significance. Antibody measurements for oxLDL also revealed the same trend, with LDLr.*Sle3* mice producing more antibodies than control (Figure 11C).

Evaluation of anti-β2GP1 and anti-cardiolipin immunoglobulin isotype titers demonstrated significantly higher IgG1 and IgM, but not IgG2c, titers in the sera of LDLr.*Sle3* mice compared to control mice. Calculation of the IgG1 (Th2) to IgG2c (Th1) isotype ratio demonstrated that LDLr.*Sle3* mice had an increased IgG1/IgG2c ratio (Table 5). We then went on to analyze the levels of IFN-γ and IL-4 in the sera of these mice and found significantly less IFN-γ levels ($p=0.036$) in LDLr.*Sle3* mice (35.22pg/ml; n=8) compared to LDLr.B6 controls (66.33pg/ml; n=8). IL-4 was not different between the two groups (data not shown). Taken together, these results show that the LDLr.*Sle3* mice have increased auto-antibody production compared to control animals and that hematopoietic transfer of the *Sle3* lupus-susceptibility loci is sufficient to exacerbate humoral immune responses against atherosclerosis-associated antigens, such as β2GP1 and oxLDL.

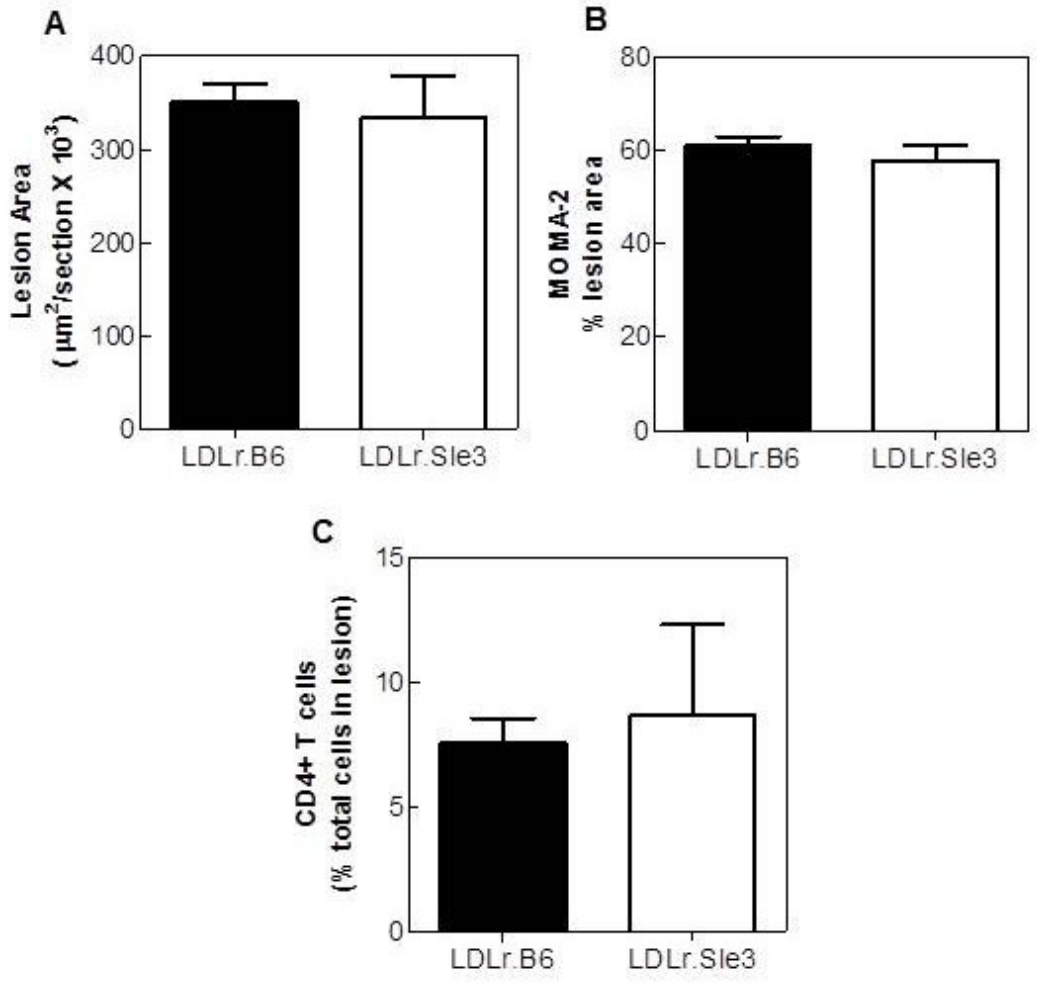

Figure 9. Analysis of atherosclerosis and cellular composition in the aortic sinus. (A) Average lesion area as determined by Oil-Red-O staining. N=17-19 mice per group **(B)** Immunohistochemical detection of macrophages (MOMA-2) and **(C)** $CD4^+$ T cells. In all panels closed bars represent LDLr.B6 mice while open bars represent LDLr.*Sle3* mice. For B and C, n=3 mice per group.

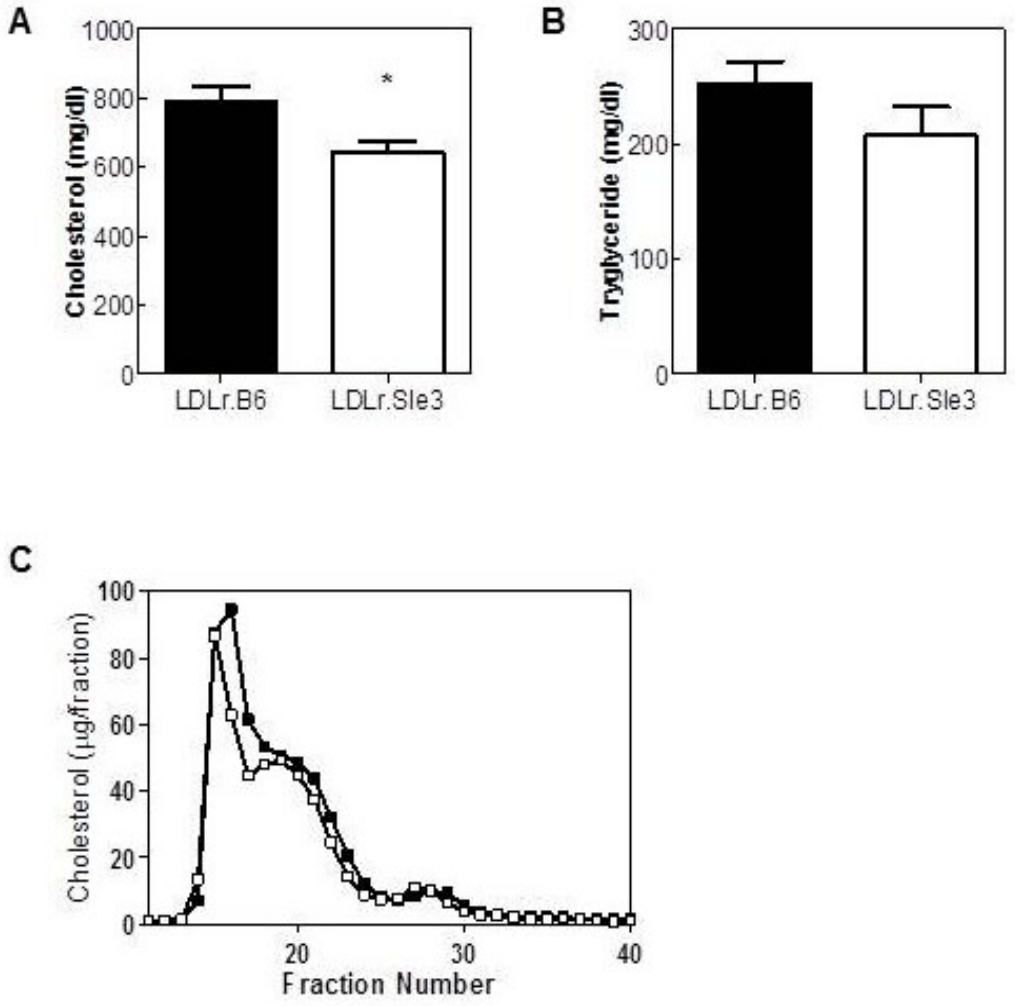

Figure 10. Transfer of *Sle3* to LDLr$^{-/-}$ mice decreases serum cholesterol and triglyceride levels. **(A)** Serum cholesterol (p=0.013) and **(B)** triglyceride levels (p=0.159) in LDLr.B6 (closed bars) and LDLr.Sle3 (open bars) mice. For both analyses, n=17-19 mice per group. **(C)** FPLC analysis of cholesterol lipoprotein distribution in LDr.B6 (closed bars) and LDLr.Sle3 (open bars) mice. Serum from eight mice per group was pooled. * indicates a *p* value < 0.05 compared to LDLr.B6 mice.

Discussion

It is well known that individuals with SLE have an increased risk for developing CVD. Our laboratory (34, 35) and others (117), have shown that accelerated atherosclerosis can occur following hematopoietic transfer of SLE to LDLr$^{-/-}$ mice and that immune dysregulation and chronic inflammation can modulate atherosclerosis. However, the exact mechanisms mediating accelerated vascular disease in SLE are yet to be determined.

In an effort to elucidate possible mechanisms of SLE-accelerated atherosclerosis, we examined whether the *Sle3* lupus-susceptibility locus alone was sufficient to increase vascular disease in LDLr$^{-/-}$ mice. *Sle3* was found to mediate T cell and antigen presenting cell hyperactivity (108, 149, 150). In the present study, we hypothesized that T cell dysregulation, mediated by *Sle3*, is sufficient to accelerate atherosclerosis. However, we found no differences in proximal aortic lesion sizes or cellular composition between LDLr.B6 and LDLr.*Sle3*. Our results suggest that just as having one lupus susceptibility interval is not sufficient to induce fully penetrant lupus disease, 2 or more lupus susceptibility loci may be necessary to accelerate atherosclerosis in mice.

Patients with SLE produce large amounts of autoantibodies, including antibodies against atherosclerotic antigens (153, 154). In our model, analyses of humoral immune responses against such antigens demonstrated an increase in antibody production in the absence of increased atherosclerosis. When isotype specific immunoglobulin levels against β2GP1 and cardiolipin were measured, we found increased IgG1 and IgM antibodies along with an increased IgG1/IgG2c ratio in LDLr.*Sle3* mice compared to controls. These results are congruent with our previous study using LDLr.*Sle1.2.3*. mice (35) and demonstrate that while *Sle3* is not able to mediate accelerated atherosclerosis,

it is sufficient to induce humoral responses similar to those seen in the triple congenic model.

While antibody titers against atherosclerosis-associated antigens are traditionally thought to be a biomarker for cardiovascular disease risk (138, 140), reports suggest such responses can be pro- or anti-atherogenic (153). Antibodies of the IgG isotype, specific for oxLDL/ β2GP1 immune complexes are postulated to be proatherogenic as they may facilitate uptake of immune complexes through Fc receptors (141). However, other reports show that anti-oxLDL (mainly IgM) and anti-cardiolipin could potentially be anti-atherogenic (71, 155). In general, both IgM and IgG1 antibodies are thought to be atheroprotective. Additionally, IgG1 antibodies indicate a Th2 immune response as IL-4 facilitates B cell class switching to the IgG1 isotype. Conversely, Th1 cells, through their production of IFN-γ, facilitate IgG2c class switching, and IFN-γ is thought to be pro-atherogenic (156-158). Given these reports, our results present an interesting outcome. In this study, we find that although LDLr.B6 mice have an increased Th1 antibody response and LDLr.*Sle3* mice have increased atheroprotective antibodies, there are no differences in lesion area between the groups. Therefore, despite having more possibly atheroprotective antibodies, the atherosclerotic lesions develop similarly in LDLr.*Sle3* mice compared to LDLr.B6 mice. Thus it is possible that that although the *Sle3* gene locus is not enough to push the animals toward increased atherosclerosis, it does decrease the protective phenotype or functions of atherosclerosis-associated antibodies. It is possible that addition of the *Sle1 or Sle2* loci would be enough to increase autoimmune dysregulation and increase atherosclerosis in this model. Obviously this hypothesis is the focus of future investigations by our laboratory and will allow us to ascertain how epistatic interaction between these loci may affect CVD.

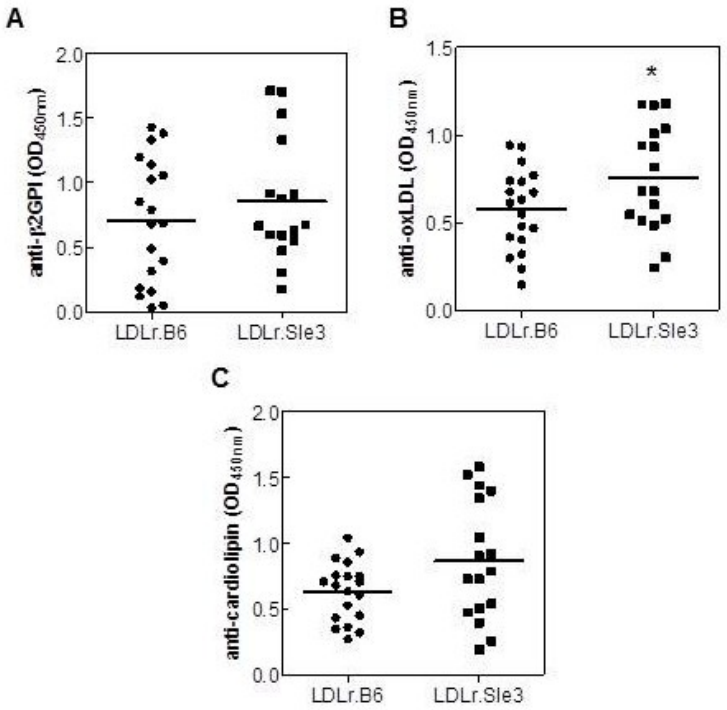

Figure 11. Antibody production is increased in LDLr.*Sle3* mice. Serum titers of **(A)** anti-β2GP1 (*p*=0.356), **(B)** anti-cardiolipin (*p*=0.053) and **(C)** anti-oxLDL (*p*=0.049) antibodies in LDLr.B6 (n=19) and LDLr.*Sle3* (n=17) mice. * indicates a *p* value < 0.05 compared to LDLr.B6 mice.

Table 5.

Serum titers of isotype specific antibodies

Group	IgG1 (Average OD ± S.E.M.)	IgG2c (Average OD ± S.E.M.)	IgM (Average OD ± S.E.M.)	IgG1/IgG2c (Average OD ± S.E.M.)
β2-GP1[a]				
LDLr.B6	0.291 ± 0.041	0.426 ± 0.040	0.470 ± 0.051	0.692 ± 0.078
LDLr.Sle3	0.525 ± 0.083[b]	.556 ± 0.083	0.829 ± 0.108[b]	1.064 ± 0.143[b]
Cardiolipin[a]				
LDLr.B6	0.384 ± 0.040	0.438 ± 0.028	0.643 ± 0.041	0.863 ± 0.071
LDLr.Sle3	0.601 ± 0.072[c]	0.487 ± 0.049	0.778 ± 0.038[b]	1.255 ± 0.111[c]

[a] 16-19 mice per group.

[b] p< 0.05 compared to LDLr.B6 mice.

[c] p< 0.001 compared to LDLr.B6 mice.

Parallel with our previous studies, we also observed decreased serum cholesterol and triglyceride levels in LDLr.*Sle3* mice compared to control animals. (34, 35) This is an interesting observation since it suggests that either genes encoded on the *Sle3* chromosomal interval are playing a role in cholesterol homeostasis in the LDLr.*Sle3* mice, or that increased anti-oxLDL and anti-phospholipid antibody levels in the lupus-susceptible mice increase uptake and/or clearance of circulating lipoproteins. We hypothesize the latter because cholesterol and triglyceride levels are similar between LDLr.B6 and LDLr.*Sle3* mice before the initiation of Western diet and the appearance of dsDNA antibodies; the first biomarker of lupus-associated immune dysregulation. Again, we find that despite lower cholesterol levels, our LDLr.*Sle3* mice have similar atherosclerotic lesion area compared to controls. These data lend further support to the hypothesis that autoimmunity rather than traditional risk factors, such as elevated cholesterol levels, is the primary mediator of accelerated atherosclerosis in the autoimmune disease.

Previously, our laboratory reported a three-fold increase in $CD4^+$ T cell burden in the atherosclerotic plaques of LDLr.*Sle1.2.3* mice (34, 35). This led us to hypothesize that the increase in vascular disease in these mice may be mediated primarily by T cell hyperactivity. Our recent findings suggest that while there is evidence of a hyperactive T cell phenotype in LDLr.*Sle3* mice (Figure 8C-E), *Sle3* by itself is not enough to initiate $CD4^+$ T cell infiltration into atherosclerotic plaques. Furthermore, the data also indicate that T cell accumulation in lesions may be one driving force of lesion progression in LDLr.*Sle1.2.3* mice as the LDLr.B6 and LDLr.*Sle3* mice had similar amounts of T cell percentages within the lesion. Indeed this is a plausible hypothesis as Zhou *et al.* found that transfer of $CD4^+$ T cells into $ApoE^{-/-}$scid/scid mice drastically increased lesion formation and that these T cells also homed to the atherosclerotic lesion area (159, 160).

Further studies examining the kinetics of T cell migration into lesions are necessary to determine if this is the case.

In conclusion, we have reported that transfer of the lupus susceptibility interval *Sle3* to LDLr$^{-/-}$ mice is not sufficient to accelerate the progression of atherosclerosis. It can, however, mediate antibody production against dsDNA and antigens typically associated with atherosclerosis, such as phospholipids and oxLDL. Additionally, the introduction of *Sle3* onto the LDLr$^{-/-}$ background is sufficient to mediate Th2 antibody bias, as previously seen in the triple congenic mouse model. Interestingly, our results suggest that in autoimmune disease, serum cholesterol levels may be a weak indicator of atherosclerosis progression. Future studies should be designed to study how the loss of tolerance to nuclear antigens may facilitate atherosclerosis and to determine how dyslipidemia may affect the autoimmune status of other congenic SLE mouse models. These studies will ultimately advance our knowledge regarding autoimmune-mediated atherosclerosis and lead to the development of therapeutic agents designed to treat both SLE and atherosclerosis.

CHAPTER IV

The lupus susceptibility loci *Sle1* and *Sle1.3* are not sufficient to accelerate atherosclerosis in LDLr$^{-/-}$ mice.

Introduction

In the previous chapter, we hypothesized that transfer of the lupus susceptibility interval *Sle3*, which is associated with T cell dysregulation, to LDLr$^{-/-}$ mice would be sufficient to accelerate atherosclerosis. We found that while *Sle3* did not lead to accelerated atherosclerosis, it did result in T cell hyperactivation and humoral responses associated with SACVD. Therefore, we hypothesized that perhaps a second interval, *Sle1* might synergize with *Sle3* to accelerate atherosclerosis.

Expression of the susceptibility locus *Sle1* most notably leads to the loss of tolerance to nuclear antigens. In addition to mediating this critical step in SLE pathogenesis, T cells expressing this locus also display a hyperactive phenotype consisting of increased proliferation and increased B cell cooperation (106, 107, 161-163). Further analysis of this locus has revealed that *Sle1* not only regulates T effector functions but also mediates decreased Treg numbers and functions through multiple mechanisms (82, 117, 164). Co-expression of *Sle1* and *Sle3* leads to splenomegaly, increased numbers of activated B and T cells and nephrophilic autoantibody production (165, 166), even more than expression of each locus singularly. Moreover, intracellular expression of both of these loci simultaneously is necessary to produce these phenotypes, supporting the hypothesis that epistatic interactions between SLE susceptibility loci are critical to lupus pathogenesis (167). Since we did not observe increased atherogenesis in the presence of *Sle3* alone, we sought to determine if –

similar to SLE nephritis development – co-expression of *Sle1* and *Sle3* was necessary to accelerate atherosclerosis in LDLr$^{-/-}$ mice.

Materials and Methods

Mice. C57Bl/6J (B6) and mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and a breeding colony maintained in the Vanderbilt University animal care facility. Female B6.129S7-Ldlrtm1Her/J (LDLr$^{-/-}$) mice used as bone marrow recipient mice were purchased from The Jackson Laboratory. The B6.NZMc1 (B6.*Sle1*), B6.NZMc7 (B6.*Sle3*) and B6.NZMc1 | c7 (B6.*Sle1.3*) mice are C57Bl/6 mice single or bi congenic for NZM2410-derived lupus susceptibility intervals and have been extensively characterized (106-108, 149, 161-163, 167, 168). These mice were a generous gift from Edward Wakeland at the University of Texas Southwestern Medical Center. All procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Production of radiation chimeras. Transfer of bone marrow was accomplished by bone marrow transplantation as previously described (34). Female LDLr$^{-/-}$ mice received either C57Bl/6, B6.*Sle1*, B6.*Sle3* or B6.*Sle1.3* bone marrow, hereafter referred to as LDLr.B6, LDLr.*Sle1*, LDLr.*Sle3* and LDLr.*Sle1.3*, respectively.

Atherosclerosis studies. Sixteen weeks after transplantation, mice were placed on a high-fat Western diet (21% milk fat, 0.15% cholesterol) for eight weeks. For chow diet studies, mice were kept on chow diet for 32 weeks after bone marrow transplantation. Mice were then sacrificed and analyzed for the degree of atherosclerosis and the presence and severity of symptoms associated with SLE.

Serum Lipoprotein Analysis. Total serum cholesterol and triglyceride were measured in mice fasted for at least four hours using a colorimetric assay as described previously (151).

Serum Enzyme-Linked Immunosorbent Assays (ELISAs). Serum titers of dsDNA were measured according to the method of Shivakumar et al. (152). Anti-oxLDL antibodies were measured as described previously (69). Serum antibody titers against β2-glycoprotein I (β2GP1) were measured by coating a 96-well Maxisorb plate with 10 μg/ml of purified β2GP1 in 1% bovine serum albumin (1% BSA)/PBS overnight. Plates were blocked in 1% BSA/PBS for two hours at room temperature. Mouse serum was added at a dilution between 1:500 and 1:5000 and incubated overnight at 4°C. Plates were washed with 0.5% Tween-20/PBS (PBS-T) and incubated with biotin-conjugated goat anti-mouse Ig(H+L) (SouthernBiotech, Birmingham, AL) for 45 minutes at room temperature then incubated with avidin-peroxidase for 30 minutes at room temperature. Plates were then washed with PBS-T and developed using TMB substrate (BD Bioscience). Anti-oxLDL immunoglobulin isotype ELISAs were performed as described above using a biotin-conjugated goat anti-mouse IgG1, IgG2c or IgM (SouthernBiotech) secondary antibody. Cardiolipin antibody titers were determined by coating a 96-well Maxisorb plate with cardiolipin (Sigma-Aldrich) (15μg/ml in 95% ethanol). The cardiolipin ELISA was then conducted as described above for β2GP1.

Cytokine ELISAs. Supernatant cytokine levels were measured by sandwich ELISA according to manufacturer's protocol (BD Pharmingen).

Immunohistochemistry. Staining for macrophages (MOMA-2) and CD4+ T cells was performed as described previously (34, 35). Trichrome staining was performed using a trichome staining kit (Newcome Supply; Middleton, WI). Cells were visualized and staining quantified using Image-Pro Plus software (Media Cybernetics, Bethesda, MD).

Flow Cytometry. For flow cytometric analyses, spleens were removed and processed through a 0.70 μm mesh screen. Cells were counted, resuspended in 1% bovine serum albumin (BSA) in HBSS with 0.02% sodium azide and 0.035% sodium bicarbonate, and incubated with appropriate antibodies for 40 minutes at 4°C. Cells were then washed and analyzed using a 7-laser MACSQuant Analyzer flow cytometer and FCS Express software (De Novo Software).

Intracellular cytokine staining. For intracellular cytokine staining, splenocytes were stimulated with 10ng/ml phorbol myristate acid (PMA) and ionomycin (1μg/mL) in the presence of GolgiStop or GolgiPlug for 4-5 hous at 37 °C and 5% CO_2. Cells were then stained with extracellular antibodies, washed, and fixed overnight in 2% paraformaldehyde in PBS. The following day, cells were permeabilized with Cytoperm (BD Bisciences) reagent, stained with the appropriate intracellular antibodies and analyzed by flow cytometry. For IL-17 secretion, instead of incubating splenocytes for 5 hours in the presence of GolgiPlug, cells were first stimulated with PMA and ionomycin without Golg-Plug for two hours then incubated an additional three hours in the presence of GolgiPlug.

Statistical Analyses. Statistical analyses were conducted using PRISM 5.0 software (GraphPad Software Inc., La Jolla, CA). Statistical significance between multiple groups was determined using a one way ANOVA with a Tukey multiple comparison test to calculate differences between groups. A p value of < 0.05 was considered significant.

Results

Phenotypes associated with *Sle1.3* can be transferred hematopoietically to LDLr$^{-/-}$ mice.

Hematopoietic cells from four to ten week old female B6, B6.*Sle1*, B6.*Sle3*, or B6.*Sle1.3* mice were transferred into lethally irradiated LDLr$^{-/-}$ mice. Although we have previously reported our findings on the effects of *Sle3* on lupus-accelerated atherosclerosis (see **Chapter 3**), we felt that it was important to include mice with this genotype in this study as an internal control. At the time of sacrifice, serum was collected to determine autoantibody production by ELISA. Similar to B6.*Sle1.3* mice, we found that serum from LDLr.Sle1.3 chimeras contained increased antibodies against dsDNA compared to LDLr.B6 mice (Figure 12A). Additionally, these mice exhibited splenomegaly. This splenomegaly was also seen in LDL.*Sle1* mice. However, there were no differences in body weight between any of the groups (Figure 12B and C).

Spontaneous activation of various splenocyte populations is known to occur in mice harboring the *Sle1*, *Sle3*, or *Sle1.3* lupus susceptibility loci (39, 108, 149, 161, 167). To determine if these changes also occurred in our radiation chimeras, splenocytes were incubated with a panel of fluorophore-conjugated antibodies and cell populations analyzed by flow cytometry. As seen previously, we observed increased CD4$^+$ T cell percentages in all experimental groups compared to LDLr.B6 mice with LDLr.*Sle1* and LDLr.*Sle1.3* mice having the highest percentages. (Figure 13A-B) This was also accompanied by increased T cell activation as both CD4$^+$ and CD8$^+$ T cells had increased CD69 expression (Figure 13C-D). While there were no differences in the percentage of CD19$^+$ B cells, NK cells, CD11c$^+$ or CD11b$^+$ cells, there was a trend towards an increased percentage of CD11c$^+$ and CD11b$^+$ cells in LDLr.*Sle1* mice (Figure 13C). Analysis of dendritic cell activation markers on CD11c$^+$ cells revealed that while LDLr.*Sle1* and LDLr.*Sle1*.3 had a lower percentage of CD11c$^+$ cells expressing the late marker of activation, CD80, CD11c$^+$ cells from these groups also had more cells expressing the early marker of activation CD86 (Figure 14).

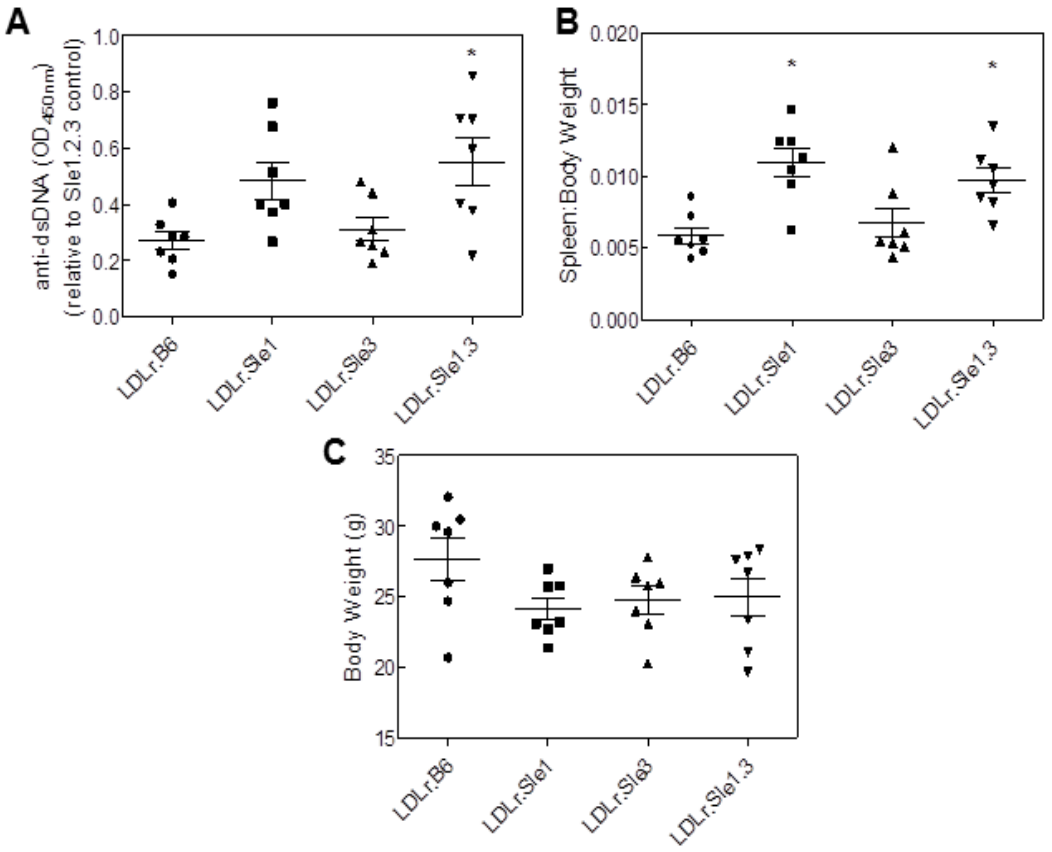

Figure 12. Characterization of lupus phenotype in single and bi-congenic SLE mice. **(A)** Serum titers of anti-dsDNA antibodies in LDLr.B6 (circles), LDLr.*Sle1* (squares), LDLr.*Sle3* (upright triangles) and LDLr.*Sle1.3* (inverted triangles) mice. **(B)** Spleen:body weight ratio of LDLr.B6 (circles), LDLr.*Sle1* (squares), LDLr.*Sle3* (upright triangles) and LDLr.*Sle1.3* mice (inverted triangles). **(C)** Body weights of LDLr.B6 (circles), LDLr.*Sle1* (squares), LDLr.*Sle3* (triangles) and LDLr.*Sle1.3* (inverted triangles) mice. * indicates a *p* value < 0.05 compared to LDLr.B6 mice. N=7-9 mice per group. Experiment was repeated twice.

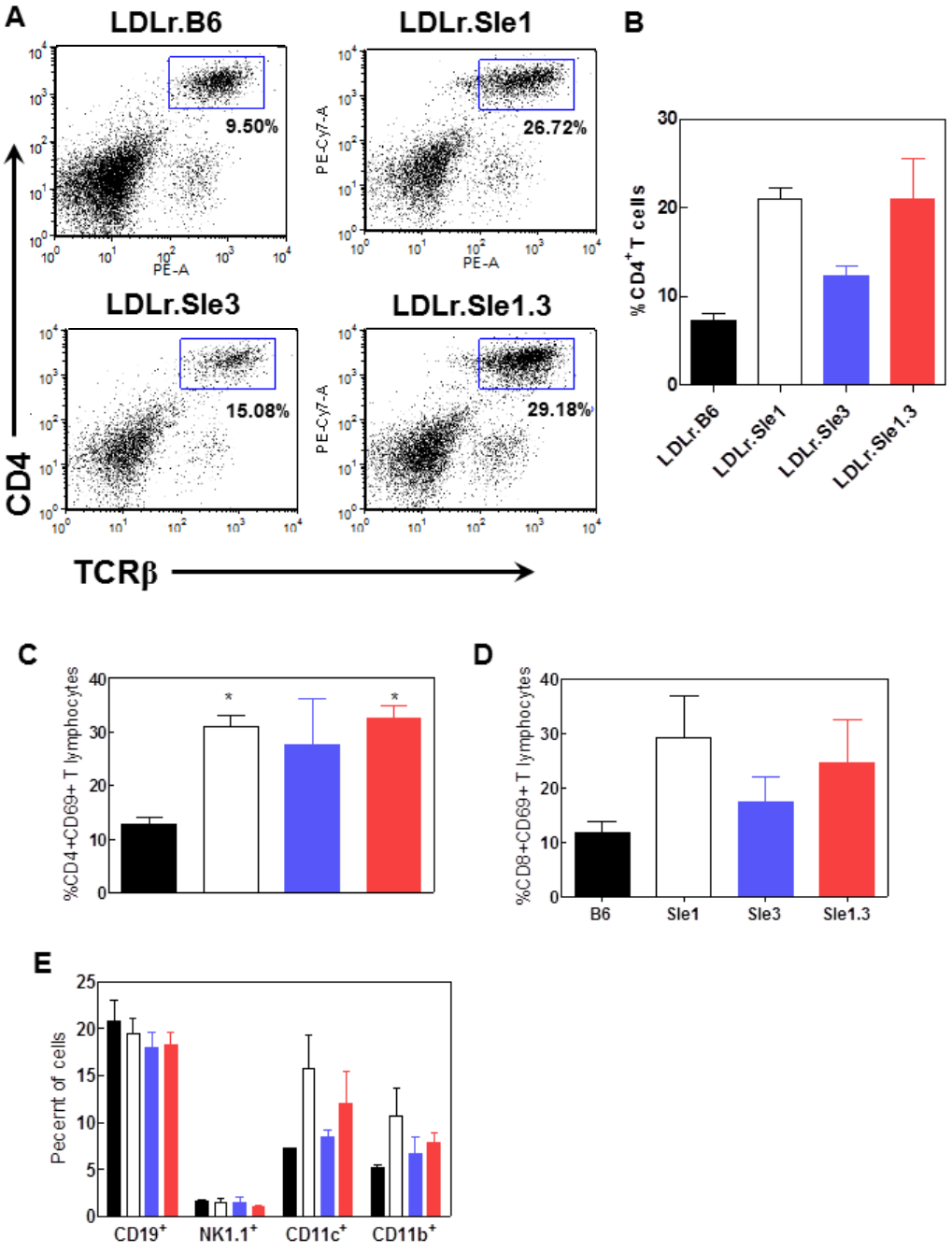

Figure 13. Analysis of splenocyte populations. (A) Representative FACS plot of $CD4^+$ T cells in LDLr.B6, LDLr.*Sle1*, LDLr.*Sle3* and LDLr.*Sle1.3* mice. **(B)** Percentage of $CD4^+$ T lymphocytes (n=3-5 mice per group). **(C)** Percentage of $CD4^+CD69^+$ T cells (n=3-5 mice per group). **(D)** Percentage of $CD8^+CD69^+$ T cells (n=3-5 mice per group). **(E)** Percentage of various cell types in mice (n=3-5 mice per group). In all bar graphs, black bar = LDLr.B6, white bar = LDLr.*Sle1*, blue bar = LDLr.*Sle3*, red bar = LDLr.*Sle1.3*. * indicates a *p* value < 0.05 compared to LDLr.B6 mice.

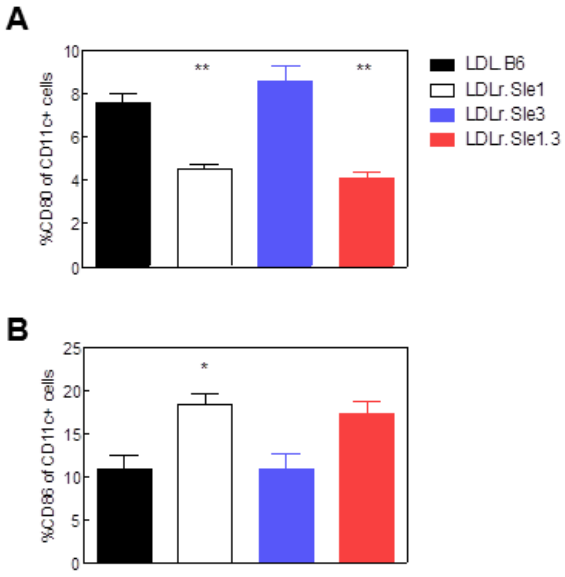

Figure 14. Effects of SLE susceptibility loci on dendritic cell activation. (A) Percentage of CD11c^{+}cells expressing CD80. **(B)** Percentage of CD11c^{+}cells expressing CD86. (n=3-5 mice per group). In both graphs, black bar = LDLr.B6, white bar = LDLr.*Sle1*, blue bar = LDLr.*Sle3*, red bar = LDLr.*Sle1.3.* * indicates a *p* value < 0.05 compared to LDLr.B6 mice. ** indicates a *p* value < 0.01 compared to LDLr.B6 mice.

The combination of the lupus susceptibility loci *Sle1* and *Sle3* mediates decreased atherosclerosis in LDLr-/- mice when placed on a high fat diet.

Sixteen weeks after transplantation, mice were placed on a high fat Western diet for eight weeks. Upon sacrifice, hearts were sectioned an atherosclerotic lesion area assessed. Measurement of atherosclerosis in the proximal aorta revealed that while there were no differences between LDLr.B6, LDLr.*Sle1* and LDLr.*Sle3* groups, the LDLr.*Sle1*.3 mice had significantly smaller atherosclerotic lesions compared to LDLr.B6 mice (Figure15A-B). In order to understand what could be responsible for this phenomenon, we examined the cellular composition of the atherosclerotic lesions of these mice. MOMA-2 staining showed no statistical differences in macrophage content of the lesions between the four groups (Figure 15C). The same was true for collagen content (assessed by Trichome staining) and CD4^{+} T cells within the lesions, suggesting

that the lack of increased T cell accumulation could be responsible for the decreased lesion area observed in LDLr.*Sle1*.3 mice.

Interestingly, we found that all experimental groups (LDLr.*Sle1*, LDLr.*Sle3* and LDLr.*Sle1*.3) had significantly less serum cholesterol levels compared to LDLr.B6 mice (Figure 16A). This trend was also true for serum triglyceride levels although only LDLr.*Sle1*.3 reached statistical significance (Figure 16B).

A Th2 bias is present in LDLr.*Sle1*.3 mice.

Antibodies against atherosclerosis associated antigens such as phospholipids and oxLDL are often used to assess atherosclerotic risk. Given that we found a decrease in atherosclerosis in LDLr.*Sle1.3* mice, we sought to determine if differences in these antigens could account for the atherosclerotic changes that we observed. Total immunoglobulin response against β2GP1, cardiolipin and oxLDL was assessed. We found no significant differences in anti- β2GP1, anti-cardiolipin or anti-oxLDL titers between any of the groups (Figure 17).

We also evaluated anti-oxLDL immunoglobulin isotypes. While there were no statistical differences in anti-oxLDL IgM, IgG1 or IgG2c titers, there was trend towards increased IgG1 and IgG2c production in LDL.*Sle1* and LDLr.*Sle1*.3 mice (Figure 18A). Calculation of the IgG1 to IgG2c isotype ratio revealed that LDLr.*Sle1*.3 had a significantly higher IgG1/IgG2c ratio, indicative of shift towards Th2 response (Figure 18B). To further verify this increased Th2 response, splenocyte populations were depleted of $CD11c^+$ and $CD19^+$ antigen presenting cells, leaving mostly T cells, and stimulated with plate bound anti-CD3 and anti-CD28 for 72 hours. After incubation, supernatants were collected and IL-4 secretion was assessed by cytokine ELISA. In concert with the findings above, we detected increased IL-4 secretion from LDLr.*Sle1*.3 cultures after stimulation compared to LDLr.B6 cultures. There was also a trend toward

increased IL-4 levels from LDLr.*Sle1* cultures but it did not reach statistical significance (Figure 18C).

LDLr.*Sle1* and LDLr.*Sle1*.3 mice have a shift in their Th17 and Treg balance.

Regulatory T cells are known to be anti-atherogenic (84) while the role of Th17 in atherosclerosis remains controversial (95-97). We sought to determine if a shift in regulatory T cell balance was present in our experimental mice. We performed intracellular cytokine and Foxp3 staining on spleen cells collected to evaluate the percentage of $Foxp3^+$ Tregs and Th17 cells. We observed a trend towards decreased Tregs in LDLr.*Sle1* and LDLr.*Sle1*.3 mice (Figure 19C). This was accompanied by a significant increase in Th17 cells in both groups, suggesting that lesion area is inversely correlated with splenic Th17 cell accumulation in LDLr.*Sle1*.3 mice (Figure 19A-B).

Previous studies suggest that TLR9 stimulation on antigen presenting cells promotes increased IL-6 secretion. It is thought that increased IL-6 secretion by antigen presenting cells help drive T cells toward a Th17 fate (169, 170) (see Addendum). Given that we found increased Th17 cells in our LDLr.*Sle1*.3 mice, we hypothesized that this Th17 cell expansion was due to increased TLR9 stimulation on antigen presenting cells. To test this hypothesis, we isolated both $CD11c^+$ and $CD19^+$ spleen cells from mice and stimulated these antigen presenting cells with CpG DNA, a known TLR9 agonist, or LPS (a TLR4 agonist) for 48 hours. Afterwards, supernatants were collected and IL-6 levels assessed. Interestingly, we found decreased IL-6 levels in all experimental groups compared to LDL.B6 mice, indicating that TLR9 stimulation by CpG does not induce IL-6 secretion in our model (Figure 20A) However, LPS stimulation did not affect IL-6 secretion (Figure 20B).

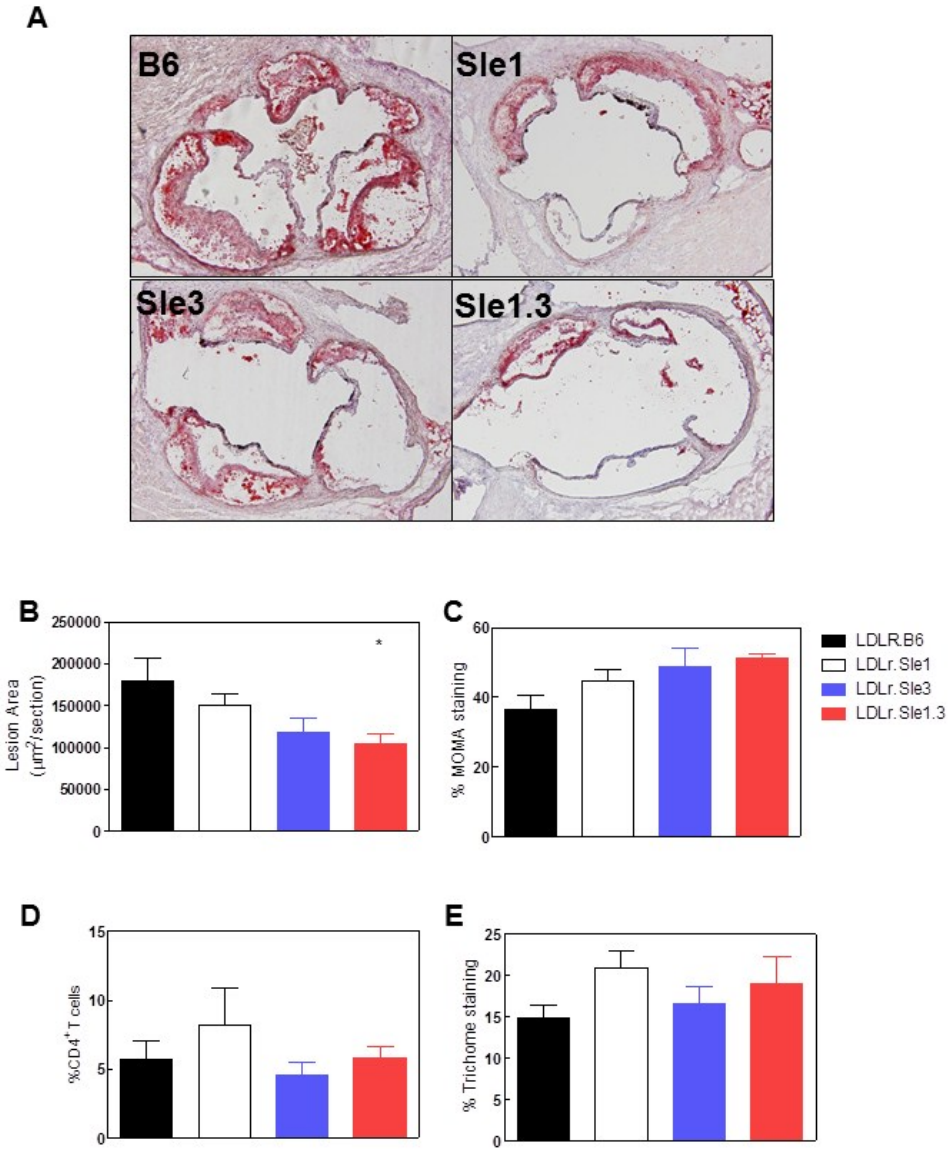

Figure 15. Analysis of atherosclerosis and cellular composition in the aortic sinus. (A) Representative aortic section stained with Oil-red-O from LDLr.B6, LDLr.*Sle1*, LDLr.*Sle3* and LDLr.*Sle1.3* mice. **(B)** Average lesion area as determined by Oil-Red-O staining. n=7-9 mice per group. **(C)** Immunohistochemical detection of macrophages (MOMA-2), **(D)** $CD4^+$ T cells, and **(E)** collagen content. N=3-5 mice per group in C, D, & E. In all graphs, black bar = LDLr.B6, white bar = LDLr.*Sle1*, blue bar = LDLr.*Sle3*, red bar = LDLr.*Sle1.3*.. * indicates a *p* value < 0.05 compared to LDLr.B6 mice.

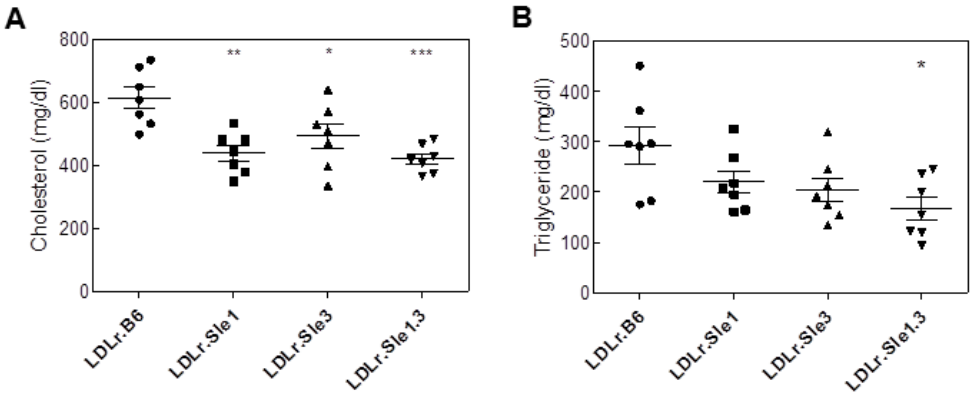

Figure 16. Serum cholesterol and triglyceride levels in single and bi-cogenic mice. (A) Serum cholesterol and **(B)** triglyceride levels in LDLr.B6 (circles), LDLr.*Sle1* (squares), LDLr.*Sle3* (upright triangles) and LDLr.*Sle1.3* (inverted triangles) mice. N=7-9 mice per group. * indicates a p value < 0.05 compared to LDLr.B6 mice. ** indicates a p value < 0.01 compared to LDLr.B6 mice. *** indicates a p value < 0.005 compared to LDLr.B6 mice.

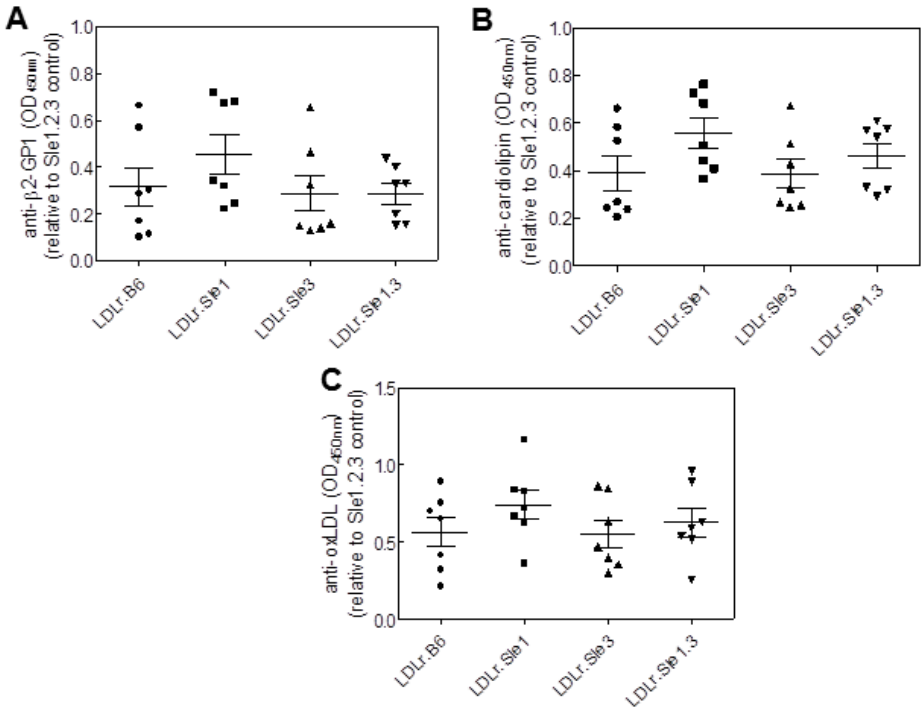

Figure 17. Phospholipid antibody production in single and bi-congenic mice. Serum titers of **(A)** anti-β2GP1, **(B)** anti-cardiolipin and **(C)** anti-oxLDL antibodies in LDLr.B6 (circles), LDLr.*Sle1* (squares), LDLr.*Sle3* (upright triangles) and LDLr.*Sle1.3* (inverted triangles) mice. N=7-9 mice per group.

There is no difference in atherosclerosis when lupus susceptible mice are kept on a normal chow diet. Previously, studies from our laboratory and others have shown that severe dyslipidemia induced by high fat diet feedings can have significant effects on both atherosclerosis and SLE (35, 128, 129). Particularly in our LDLr.*Sle1.2.3* model we found that keeping mice on chow diet led to a greater fold increase in atherosclerotic lesion area compared to those mice placed on high fat diet (refer to **Chapter 2**). Given our rather surprising result of decreased atherosclerosis in high fat diet fed LDLr.*Sle1*.3 mice, we thought it would be interesting to examine if there were changes in atherosclerosis when SLE susceptible mice are not overtly dyslipidemic.

After hematopoietic cell transfer, mice were kept on a standard chow diet for 32 weeks total. After sacrifice, proximal aortic lesion area was assessed as usual by Oil-Red O staining. We found that there were no differences in lesion area between any of the groups when kept on a chow diet (Figure 21A). However, similar to previous findings, we observed significant decreased is serum cholesterol levels in all experimental groups compared to LDLr.B6 mice on chow diet (Figure 21B-C) We also assayed autoantibody production and saw a slight, yet significant, increase in anti-dsDNA titers in LDLr.*Sle1.3* mice compared to LDLr.B6 mice (data not shown). The prototypical increase in $CD4^+$ T cells was seen as well (data not shown). Additionally, there was an increase in anti-β2GP1, anti-cardiolipin and anti-oxLDL titers in LDLr.*Sle1* mice; although, this increase was only significant for anti-cardiolipin (Table 6). These findings suggest that indeed high fat diet feeding can affect parameters of both SLE and atherosclerosis in our mice. However, even on chow diet, the combination of *Sle1* and *Sle3* is not sufficient to accelerate atherosclerosis in atherosclerosis-susceptible mice.

Discussion

In this chapter, we set out to determine if the break in tolerance to nuclear antigens is necessary to accelerate atherosclerosis in SLE, as the minimal genetic interval in B6.*Sle1.2.3* mice necessary to exacerbate vascular disease is not known. In the previous chapter, we demonstrated that transfer of *Sle3* alone to LDLr$^{-/-}$ mice was not sufficient to exacerbate atherosclerosis. However, these studies did not determine whether *Sle3* was necessary to enhance atherosclerosis in lupus. Given the increased T cell burden in our LDLr.*Sle1.2.3* mice and since *Sle1* and *Sle3* are mainly responsible for T cell dysregulation, this study was designed to examine the effects of both loci, both coupled and singularly, on atherosclerosis progression.

Contrary, to our hypothesis, we found that the presence of *Sle1* and *Sle3* together in LDLr$^{-/-}$ mice led to a decrease in atherosclerosis when mice were placed on Western diet. This is a surprising finding for us given that in most of our analyses, LDLr.*Sle1*.3 mice displayed the highest amount of immune hyperactivity. The most straightforward explanation for this conundrum would be that decreased circulating lipoprotein levels are responsible for the decrease in atherosclerosis we observed. This suggests that expression lupus susceptibility loci *Sle1* and *Sle3* by hematopoietic cells can modulate cholesterol homeostasis relative to LDLr.B6 mice (even in the presence of autoimmunity). This may indeed be the case; however, even in the presence decreased serum lipoprotein levels in LDLr.*Sle1.2.3* mice we observe increased atherosclerosis. Moreover, when we normalize plaque area to cholesterol levels we find no correlation between circulating lipoprotein levels and lesion area. Therefore are more likely scenario is that in single and bi-congenic mice, the lack of increased T cell accumulation in the lesion is responsible for the differences in lesion area. Studies using other mouse models of SACVD have reported similar drops in cholesterol levels suggesting that in the

setting of autoimmunity, immune dysregulation is paramount to elevated cholesterol when assessing cardiovascular disease risk.

Parallel with published studies we found that the presence of *Sle1* and/or *Sle3* loci was associated with a Th2 bias in LDLr$^{-/-}$ mice, as our experimental groups had an increased IgG1 to IgG2c antibody ratio against oxLDL, an antigen linked to cardiovascular disease risk (Figure 18). SLE is considered to be a largely Th2 mediated disease therefore these results are not surprising. Nonetheless, this is an intriguing observation as it could imply that without all 3 lupus susceptibility loci present, the Th2 environment established in LDLr.*Sle1*.3 mice may result in Th2 mediated athero-protective phenotypes associated with traditional atherosclerosis. Furthermore, as alluded to in **Chapter 3**, the increased IgG1 anti-oxLDL antibody titers seen in LDLr.*Sle1*.3 mice could facilitate increased clearance of circulating lipoproteins, adding to the atheroprotective phenomenon we observe. These interpretations definitely warrant future studies.

In an effort to rule out the possibility of severe dyslipidemia masking the effects of our lupus susceptibility loci on SACVD, we initiated a parallel study in which experimental mice were kept on a chow diet instead of being switched to Western diet. We found that on chow diet there were no differences in atherosclerosis between any of the groups. Additionally, we found that all experimental groups had half the amount of serum cholesterol than LDLr.B6 mice (Figure 21). This finding also suggests that cholesterol levels serve as a weak indicator of cardiovascular risk in SLE and that lesional T cell accumulation in responsible for the accelerated atherosclerosis observed in LDLr.*Sle1.2.3* mice. It is important to note that in these studies mice were fed chow diet for 32 weeks, while in our Western diet studies mice were switched to Western diet sixteen weeks after hematopoietic cell transplantation and kept on diet for 8 weeks, giving the Western diet-fed mice a chimeric life span of 24 weeks. Thus it is possible that

even without severe dyslipidemia, by allowing atherogenesis to progress for an additional eight weeks, we could have veiled any changes that may have been apparent if we had sacrificed the mice at the same time point of our Western diet studies. This is a plausible explanation as we note in **Chapter 2** there was an increased fold change in lesion area seen in LDLr.*Sle1.2.3* and LDLr.B6 mice kept on chow diet versus those on Western diet for the same time period. Also, premature cardiovascular events are linked with SLE in humans indicating that understanding early atherosclerotic events may be more important when evaluating SACVD.

Finally, we are aware that some of the trends in our analyses from **Chapter 3** were not seen when we repeated the studies using LDLr.*Sle3* mice in **Chapter 4**. Our most plausible explanation lies in the difference between the mice used for these studies. In **Chapter 3**, all of the LDLr$^{-/-}$ bone marrow recipient mice used were bred in our animal facility at Vanderbilt University. In **Chapter 4**, we purchased all of our bone marrow recipient mice from The Jackson Laboratory, as it was more efficient given the large number of mice needed for the study. Recently a study by the Hazen laboratory examined the role of gut flora in promoting cardiovascular disease (171). They found that gut flora from apoE$^{-/-}$ mice played a critical role in metabolizing dietary choline, promoting diet-induced atherosclerosis when mice were placed on a choline-rich diet. Elimination of commensal gut flora through broad spectrum antibiotic treatment led to an inhibition of diet-induced atherogenesis. Moreover, the Mathis group has also reported that commensal gut flora is important for the pathogenesis of autoimmune rheumatoid arthritis (172). In view of the fact that the LDLr$^{-/-}$ mice (and their parents) from **Chapter 4** were initially housed in The Jackson Laboratory facility which may have different animal housing standards than Vanderbilt University facilities, it is possible that differences in gut microflora could partly explain the variances in results between the two chapters. Further investigation on the role of intestinal microbiota on atherosclerosis and

autoimmunity are needed to verify this claim. Nevertheless, since all four groups of mice compared in this study came from the same institution, we believe that the results reflected here are accurate.

In summary, here we report that the loss of tolerance to nuclear antigens in addition to T cell hyperactivity, mediated my *Sle1* and *Sle3*, is not sufficient to accelerate atherosclerosis. These results indicate that all three lupus susceptibility loci are needed to exacerbate cardiovascular disease. However, this does not exclude the possibility that phenotypes associated with Sle2 may be sufficient to accelerate atherosclerosis. In addition, results presented here lead us to consider the fact that peripheral T cell hyperactivity alone does not drive atherogenesis and that there are other factors involved in the migration and accumulation of T cells within the plaques of LDLr.*Sle1.2.3* mice. In any case, these results demonstrate that further studies are warranted to ascertain how these genetic loci modulate SACVD development.

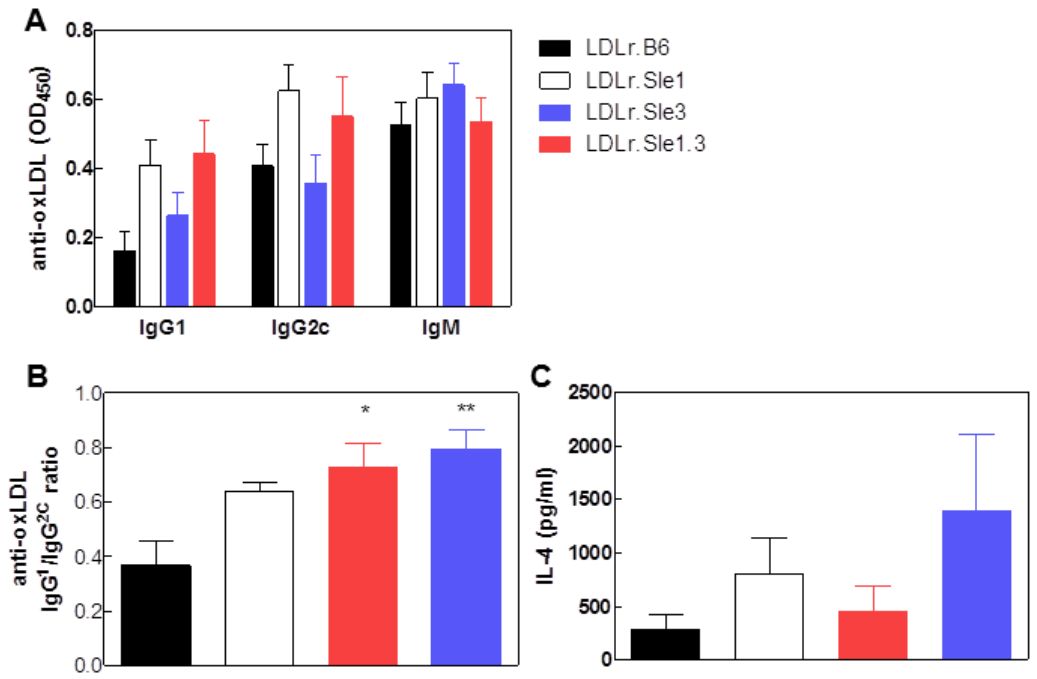

Figure 18. A Th2 bias is present in LDLr.*Sle1.3* mice. (A) Serum isotype titers of anti-oxLDL in LDLr.B6, LDLr.*Sle1*, LDLr.*Sle3* and LDLr.*Sle1.3* mice. **(B)** Anti-oxLDL IgG1/IgG2c isotype ratio. **(C)** Supernatant IL-4 secretion in mice. In all panels, black bar = LDLr.B6, white bar = LDLr.*Sle1*, blue bar = LDLr.*Sle3*, red bar = LDLr.*Sle1.3*. In A-C, n=3-5 mice per group. * indicates a *p* value < 0.05 compared to LDLr.B6 mice. ** indicates a *p* value < 0.01 compared to LDLr.B6 mice.

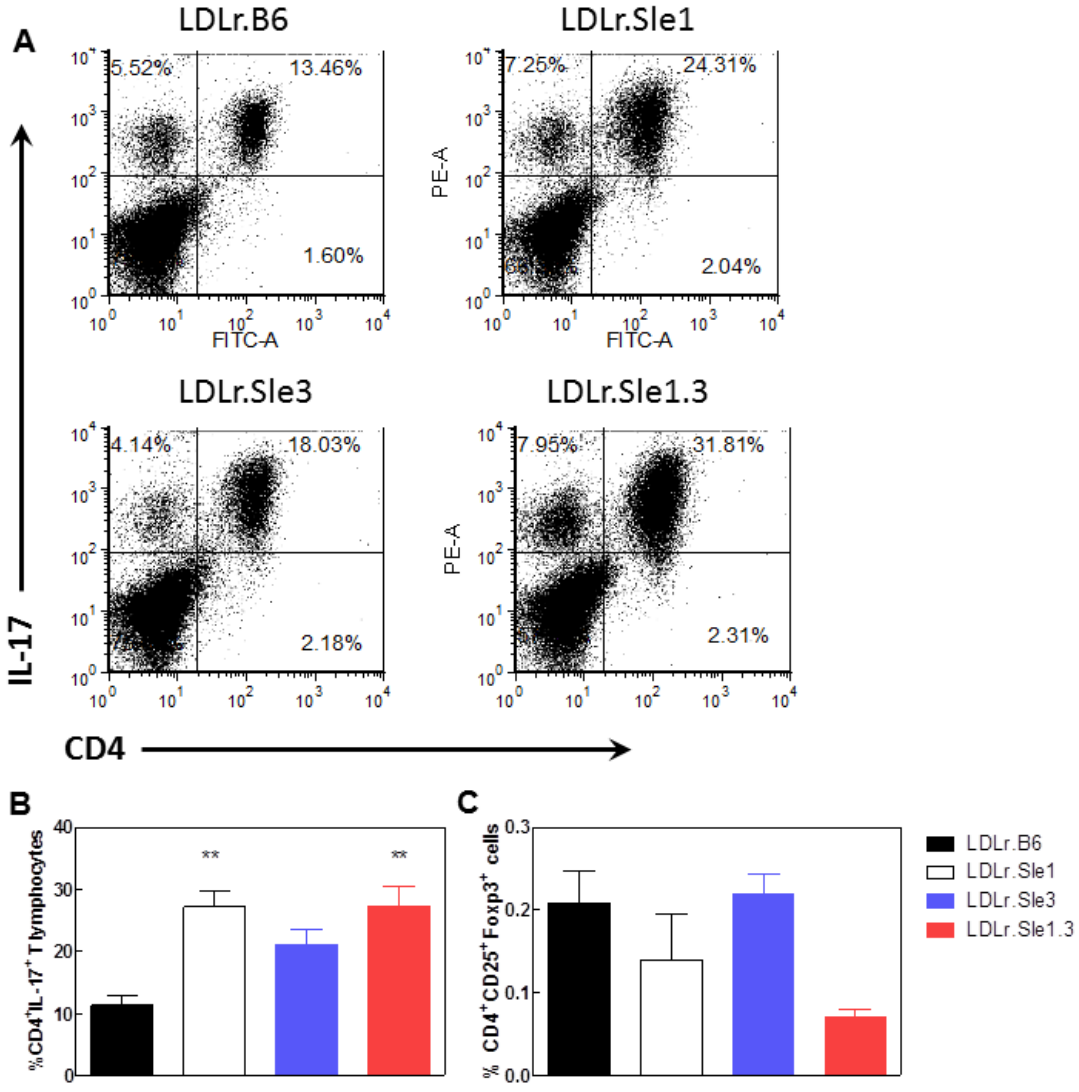

Figure 19. Increased Th17 and decreased Tregs in experimental groups. (A) Representative FACS plot of $CD4^+IL\text{-}17^+$ T cells in LDLr.B6, LDLr.*Sle1*, LDLr.*Sle3* and LDLr.*Sle1.3* mice (Gated on $TCR\beta^+$ cells). **(B)** Percentage of $CD4^+IL\text{-}17^+$ T lymphocytes (n=3-5 mice per group). **(C)** Percentage of $CD4^+CD25^+Foxp3^+$ T cells (n=3-5 mice per group). In all bar graphs, black bar = LDLr.B6, white bar = LDLr.*Sle1*, blue bar = LDLr.*Sle3*, red bar = LDLr.*Sle1.3*. ** indicates a *p* value < 0.01 compared to LDLr.B6 mice. Experiment repeated at least twice with comparable results.

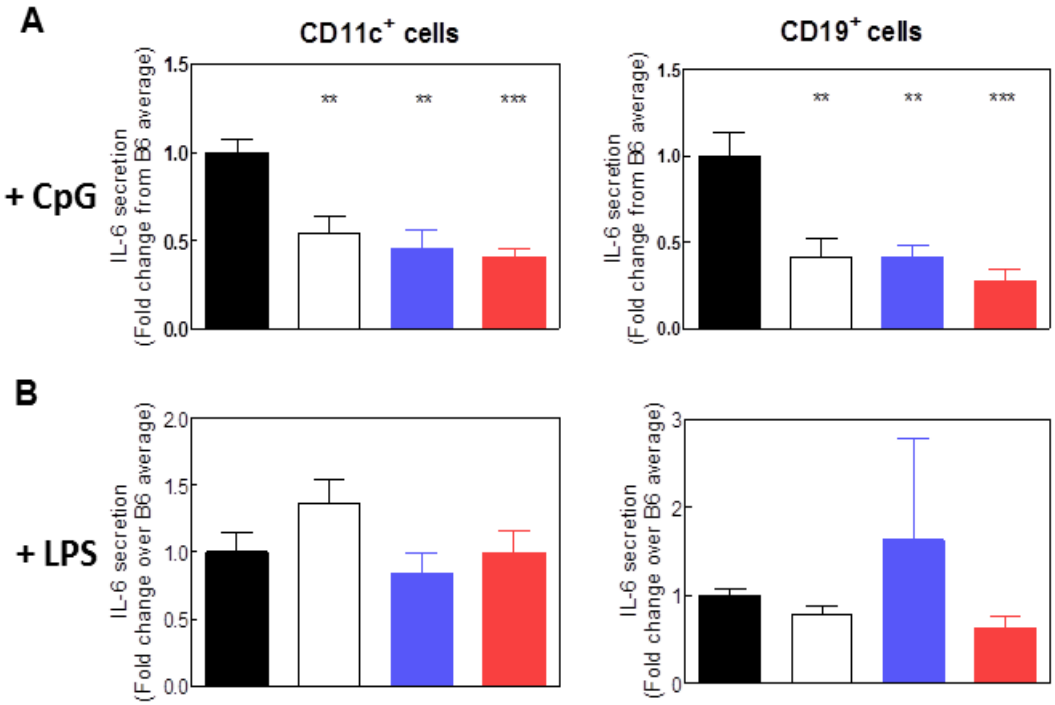

Figure 20. TLR signaling in experimental mice. IL-6 production by $CD11c^+$ (left panel) or $CD19^+$ (right panel) cells after CpG **(A)** or LPS **(B)** stimulation (n= 6-7 mice per group). In all bar graphs, black bar = LDLr.B6, white bar = LDLr.*Sle1*, blue bar = LDLr.*Sle3*, red bar = LDLr.*Sle1.3.* * indicates a p value < 0.05 compared to LDLr.B6 mice. ** indicates a *p* value < 0.01 compared to LDLr.B6 mice. *** indicates a *p* value < 0.005 compared to LDLr.B6 mice.

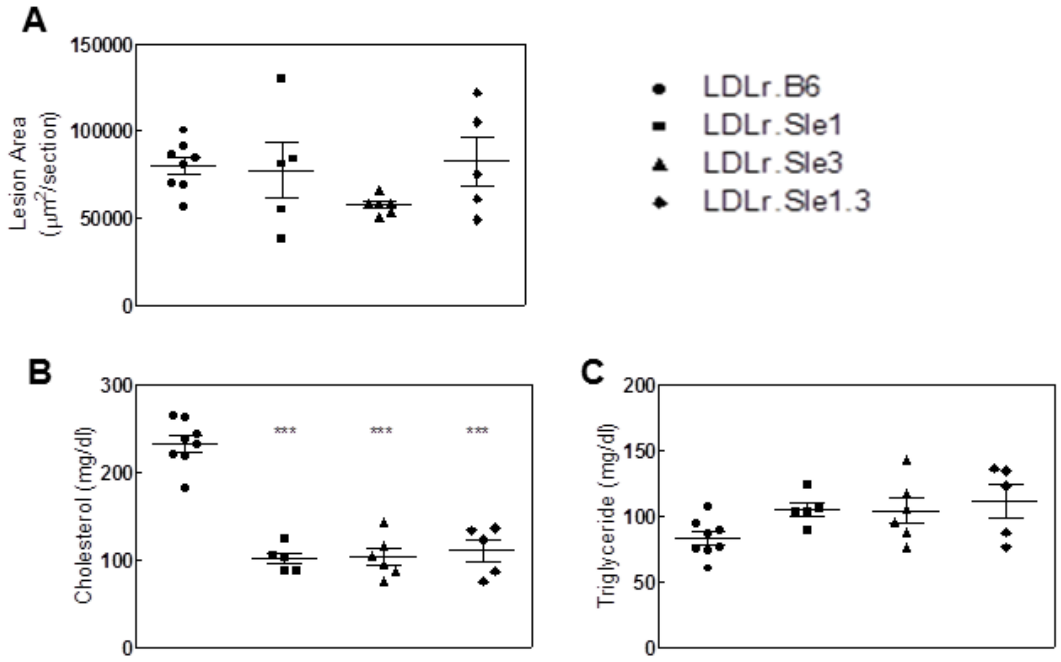

Figure 21. Effects of chow diet feeding on atherosclerosis in experimental SLE mice. (A) Average lesion area as determined by Oil-Red-O staining in LDLr.B6 (circles), LDLr.*Sle1* (squares), LDLr.*Sle3* (upright triangles) and LDLr.*Sle1.3* (inverted triangles) mice. **(B)** Serum cholesterol and **(C)** triglyceride levels. *** indicates a *p* value < 0.005 compared to LDLr.B6 mice. For A-C, n=5-8 mice per group.

Table 6.

Serum titers of auto-antibodies

Group[a]	**anti-dsDNA** (Average OD ± S.E.M.)	**anti-β2GP1** (Average OD ± S.E.M.)	**anti-oxLDL** (Average OD ± S.E.M.)	**anti-cardiolipin** (Average OD ± S.E.M.)
LDLr.B6	0.067 ± 0.0087	0.030 ± 0.0060	0.298 ± 0.0347	0.067 ± 0.0080
LDLr.*Sle1*	0.233 ± 0.0598	0.064 ± 0.0166	0.500 ± 0.0835	0.179 ± 0.0340
LDLr.*Sle3*	0.139 ± 0.0178	0.035 ± 0.0077	0.321 ± 0.0455	0.144 ± 0.0323
LDLr.*Sle1.3*	0.423 ± 0.2018[b]	0.043 ± 0.0096	0.328 ± 0.1093	0.130 ± 0.0273

[a] 5-8 mice per group.

[b] p< 0.05 compared to LDLr.B6 mice.

CHAPTER V[†]

Transfer of *Sle1.2.3* T cells is sufficient to accelerate atherosclerosis in $Rag^{-/-}LDLr^{-/-}$ mice.

INTRODUCTION

T cells are known to be pro-atherogenic under classical atherosclerosis settings. In mice, absence of T and B lymphocytes through recombinase activating gene (RAG) deficiency or severe combined immune deficiency was shown to decrease atherogenesis. Moreover, transfer of $CD4^+$ T lymphocytes into immunodeficient $apoE^{-/-}$ mice resulted in an almost two–fold increase in lesion area. (159, 160) In humans, it has also been shown that T cell accumulation is inversely associated with plaque stability (173, 174). These and other studies underscore the importance of T cells in mediating disease.

Sle1.2.3 expression on mouse T cells is associated with T cell hyperactivity (39, 107). In our mouse model of SLE accelerated atherosclerosis, we observe increased T cell accumulation within the atherosclerotic plaque (34, 35) and this increased T cell accumulation has also been seen in another model of SACVD, suggesting that in the setting of lupus, T cells play an important role in mediating enhanced vascular disease (32). While the previous chapters in this discourse focused on determining the minimal genetic interval necessary to induce changes in CVD progression, in this chapter we shift our focus to examine the cellular compartments needed to exacerbate atherosclerosis in LDLr.*Sle1.2.3* mice. The goals of these studies were to (1) determine if *Sle1.2.3* expression on T cells is necessary to induce changes in atherosclerotic

[†] Certain data in this chapter were published in the reference (53). Data from this reference will be denoted by a "†".

disease progression and (2) examine how modulation of T cell compartment can affect CVD and SLE pathogenesis.

Materials and Methods

Mice. C57Bl/6 (B6) mice were originally obtained from The Jackson Laboratory and are maintained in our colony. The SLE congenic B6.*Sle1.2.3* strain has been described and characterized extensively (37-39, 105-108). $Rag^{-/-}LDLr^{-/-}$ mice were generated in our laboratory by crossing B6.129S7-Rag1tm1Mom/J mice (obtained from The Jackson Laboratory) to $LDLr^{-/-}$ mice (maintained in our colony) and screening for the absence of both genes. All mice are maintained and used according to the guidelines and the approval of the Vanderbilt University Institutional Animal Care and Use Committee.

Purification of T cells. To isolate $CD4^+$ T cells, spleens were passed through a 0.70μm mesh screen to create a single cell suspension. $CD4^+$ T cells were isolated using $CD4^+$ magnetic beads according to Miltenyi Biotecs positive selection cell sorting protocol. Purity was assessed by flow cytometry and cell found to be ~90% enriched for CD4 expression.

Adoptive transfer of T cells. Female $Rag^{-/-}LDLr^{-/-}$ mice were sublethally irradiated (450 rads). Mice received $7X10^6$ $CD4^+$ T cells from B6 or B6.*Sle1.2.3* mice via the retro-orbital venous plexus.

Atherosclerosis studies. Two weeks after T cell transfer, mice were placed on a high-fat Western diet (21% milk fat, 0.15% cholesterol) for ten weeks. Mice were then sacrificed and analyzed for the degree of atherosclerosis and the presence and severity of symptoms associated with SLE.

† MMF treatment studies. Lethally irradiated female $LDLr^{-/-}$ mice were transplanted with bone marrow from either lupus-susceptible B6.*Sle1.2.3* (LDLr.Sle) or C57BL/6

(LDLr.B6) mice as previously described. At 16 weeks after transplantation, LDLr.B6 mice and LDLr.*Sle1.2.3* mice were placed on a Western diet (20% milk fat, 0.15% cholesterol) containing either no treatment (control) or MMF (40 mg/kg/day) for 8 weeks. After 8 weeks mice were sacrificed and analyzed for parameters of SLE and atherosclerosis.

Flow Cytometry. For flow cytometric analyses, spleens were removed and processed through a 0.70 μm mesh screen. Cells were counted, resuspended in 1% bovine serum albumin (BSA) in HBSS with 0.02% sodium azide and 0.035% sodium bicarbonate, and incubated with appropriate antibodies for 40 minutes at 4°C. Cells were then washed and analyzed using a 7-laser MACSQuant Analyzer flow cytometer (MACS and FCS Express software (De Novo Software).

Intracellular cytokine staining. For intracellular cytokine staining, splenocytes were stimulated with 10ng/ml phorbol myristate acid (PMA) and ionomycin (1μg/mL) in the presence of GolgiStop or GolgiPlug (both from BD BoSciences) for 4-5 hours at 37 °C and 5% CO_2. Cells were stained for extracellular receptors, washed, and fixed overnight in 2% paraformaldehyde in PBS. Cells were then permeabilized with Cytoperm (BD Biosciences) reagent, stained with the appropriate intracellular antibodies and analyzed by flow cytometry. For IL-17 secretion, instead of incubating splenocytes for 5 hours in the presence of GolgiPlug, cells were first incubated with PMA and ionomycin without Golg-Plug for two hours then incubated and additional three hours in the presence of GolgiPlug.

Cytokine ELISAs. Supernatant cytokine levels were measured by sandwich ELISA according to manufacturer's protocol (BD Pharmingen).

Statistical Analyses. Statistical analyses were conducted using PRISM 5.0 software (GraphPad Software Inc., La Jolla, CA). A Student's t-test was used to calculate significant differences between groups that were normally distributed while a Mann-

Whitney U test was performed for data not normally distributed. A p value < 0.05 was considered significant.

Results

Analysis of transferred T cell populations.

To determine whether lupus T cells are sufficient to accelerate atherosclerosis, we performed adoptive transfer experiments. $CD4^+$ T cells were isolated from 6 to 9 month old B6 or B6.*Sle1.2.3* mice. These mice were screened for the presence of anti-dsDNA antibody titers, indicative of active SLE disease (Figure 22A). We found that our isolation protocol yielded a highly enriched $CD4^+$ T cell population (90-95% purity, Figure 22B). Before transfer we also assessed the phenotype of these purified T cells by flow cytometry. We found that of the B6 T cells purified over 75% of them were naïve ($CD4^+CD25^-Foxp3^-$) and 9% percent $CD25^+Foxp3^+$ Tregs. Yet twice as many of the SLE T cells were $CD25^+Fop3^+$. There was also an increased prevalence of $CD4^+CD25^-$ $Foxp3^+$ cells (Figure 22C).

7 $X10^6$ T cells were transferred into six to eight week old, sublethally irradiated $Rag^{-/-}LDLr^{-/-}$ mice. Two weeks after transplantation mice were placed on a Western diet for 10 weeks then sacrificed. At sacrifice, we found no differences in body, spleen or kidney mass between the two groups (data not shown).

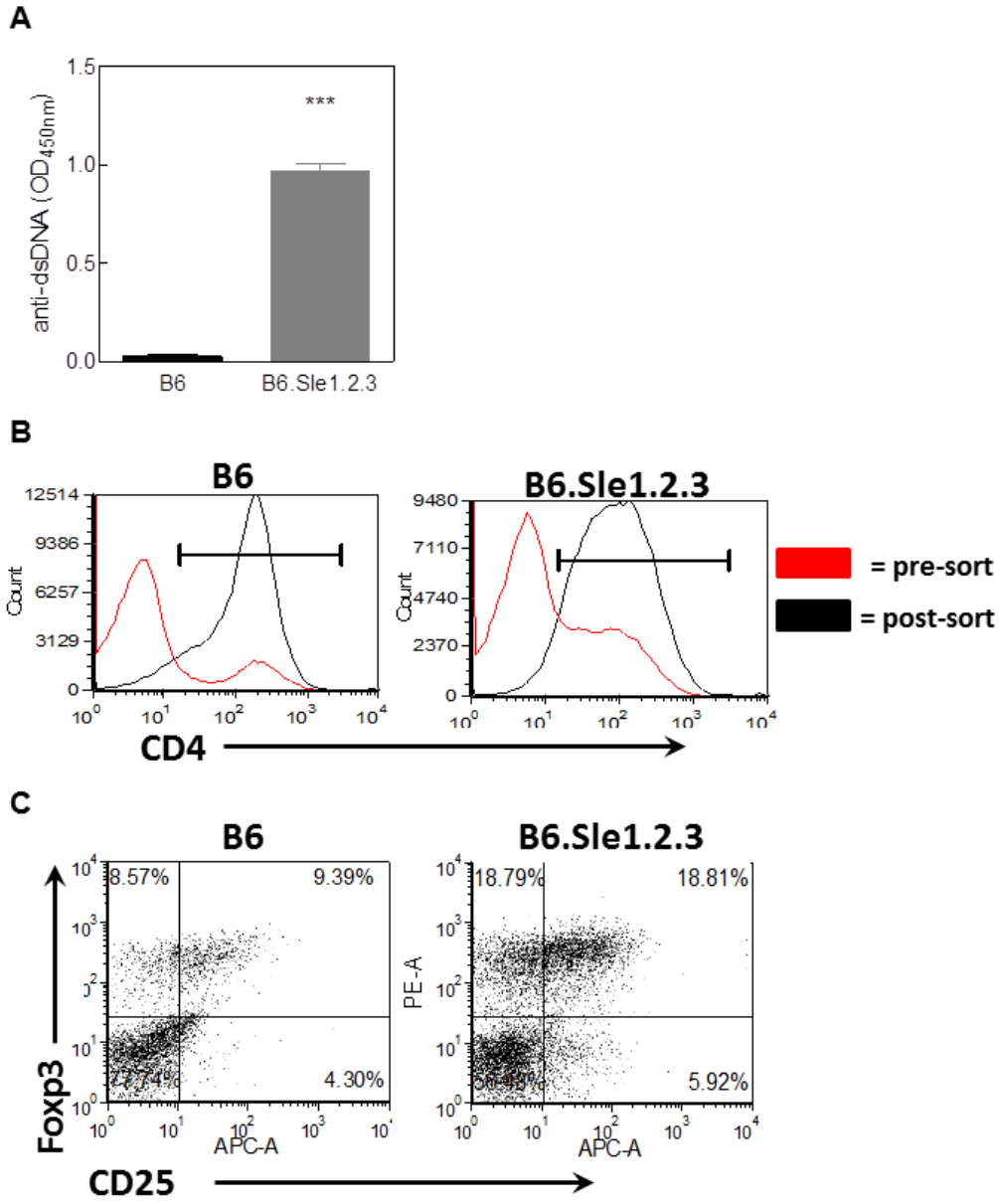

Figure 22. Analysis of CD4$^+$ population in B6 and B6.*Sle1.2.3* mice before adoptive transfer. **(A)** Anti-dsDNA antibody titers in donor B6 (black bar) and B6.*Sle1.2.3* (grey bar) mice (n=3 mice per group). **(B)** Representative histogram of CD4$^+$ expression before (red line) and after (black line) CD4-positive magnetic bead separation. **(B)** CD25 and Foxp3 expression on isolated CD4$^+$ cells before adoptive transfer into Rag$^{-/-}$LDLr$^{-/-}$ mice (gated on CD4$^+$ cells). *** indicates a *p* value < 0.005 compared to control mice.

Transfer of B6.*Sle1.2.3* T cells into Rag$^{-/-}$LDLr$^{-/-}$ mice results in increased atherosclerosis.

As a result of our previous studies which suggest that T cells are the predominant force driving SACVD in LDLr.*Sle1.2.3* chimeras, we hypothesized that transfer of B6.*Sle1.2.3* T cells from diseased mice is sufficient to accelerate atherosclerosis in Rag$^{-/-}$LDLr$^{-/-}$ mice. Analysis of Oil-Red-O staining of proximal aorta sections showed that indeed Rag$^{-/-}$LDLr$^{-/-}$ mice that received B6.*Sle1.2.3* T cells had increased lesion area compared to those with B6 T cells (Figure 23A, top panel and 23B). Moreover, the increase in lesion area was accompanied by increased lesional T cell accumulation (Figure 23A, bottom panel and 23C). Evaluation of serum lipoprotein levels confirmed that there were no differences in cholesterol or triglyceride levels suggesting that changes in atherogenesis were not due to changes in cholesterol homeostasis (Figure 24).

The effect of adoptive transfer of B6.*Sle1.2.3* T cells on splenocyte populations.

We analyzed the effects of adoptive transfer of T cells on antigen presenting cell populations. There were no differences in CD11c^{+} or CD11c^{+}CD11b^{+} cell populations, however transfer of B6.*Sle1.2.3* T cells resulted in increased CD11c^{+}B220^{+} plasmacytoid-like dendritic cells (Figure 25A). Furthermore, when we examined dendritic cell activation marker expression we found decreased expression of CD80 and CD86 on CD11c^{+} cells (Figure 25B).

Taking into account the hyperactive T cell phenotype associated with our original SACVD mouse model, we evaluated the activation status of adoptively transferred T cells in the Rag$^{-/-}$LDLr$^{-/-}$ mouse model. We found no differences in the percentage of CD4^{+} T cells between the two groups (Figure 26A). Surprisingly, there were also no differences the *ex vivo* expression of CD44 or CD69 on B6 or B6.*Sle1.2.3* CD4^{+} T cells

when isolated from $Rag^{-/-}LDLr^{-/-}$ mice (Figure 26B-C). Assessment of CD40L and CD69 expression after *in vitro* stimulation with PMA and ionomycin revealed that there were again no differences between the groups (Figure 26D-E), indicating that while transfer of B6.*Sle1.2.3* T cells can enhance atherogenesis, T cells in peripheral lymphoid organs of $Rag^{-/-}LDLr^{-/-}$ mice are not hyperactive.

To determine if differences in T helper responses could be responsible for the accelerated atherosclerosis we observed, we stimulated splenocytes and measured intracellular cytokine expression. We found no differences in expression of IL-2 or IL-17 by $CD4^+$ T cells between the groups (Figure 27A-C). There was slight increase in IFN-γ levels in mice with B6.Sle.1.2.3 T cells but this increase was not significant (Figure 27D).

†Inhibition of lymphocyte function by mycophenolate mofetil treatment ameliorates SLE and atherosclerosis pathogenesis in LDLr.*Sle1.2.3* mice.

Mycophenolate mofetil (MMF) is an immunosuppressant used commonly in organ transplantation and autoimmunity. It inhibits lymphocyte proliferation and function and studies indicate that MMF treatment is beneficial in treating both atherosclerosis and SLE. (175, 176)Given our findings that expression of *Sle* expression on T cells was sufficient to accelerate atherosclerosis, we asked if MMF would be an effective treatment for SLE and atherosclerosis in our LDLr.*Sle1.2.3* mice.

LDL.Sle.1.2.3 radiation chimeras were treated with MMF as outlined in the "Materials and Methods" section. We observed that MMF treatment significantly reduced dsDNA antibody titers (Figure 28A). Upon examination of proximal aortic lesion area, we discovered that mice treated with MMF had notably smaller lesions compared to untreated mice (Figure 28B, top panel) This decrease in lesion area also correlated with a decrease in T cell infiltration into the proximal aorta (Figure 28B, bottom panel). Flow cytometric analysis of T cell activation revealed that treatment also led to a decrease in

CD69 expression on both CD4 and CD8 T cells (data not shown). These data indicate that therapies known to modulate T cell function may prove beneficial in patients with SACVD.

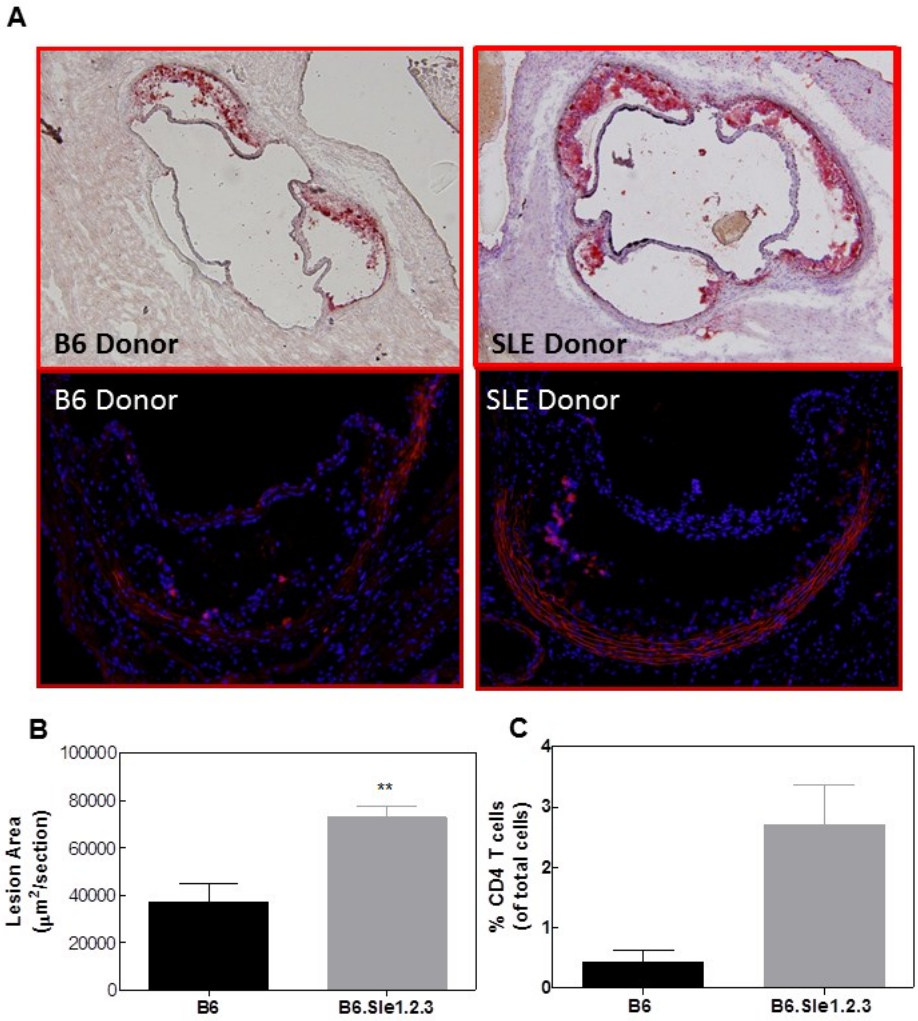

Figure 23. Transfer of B6.*Sle1.2.3* T cells accelerates atherosclerosis in Rag$^{-/-}$LDLr$^{-/-}$ mice. (A) Top panel, representative aortic sections stained with Oil-red-O. Bottom panel, representative CD4 staining. **(B)** Quantitative analysis of lesion area as assessed by Oil-red-O staining. **(C)** Percentage of CD4^{+} cells in the lesion. In B & C, n= 6 mice per group. * indicates a *p* value < 0.05 compared to control mice. ** indicates a *p* value < 0.01 compared to control mice.

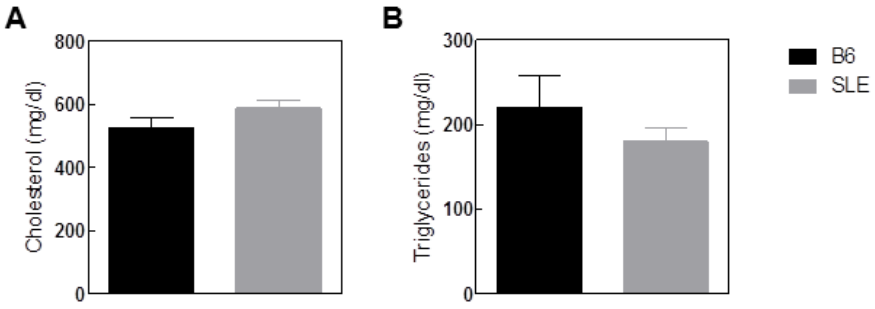

Figure 24. There are no differences in serum lipoprotein levels after adoptive transfer of CD4$^+$ T cells. Serum cholesterol **(A)** and triglyceride **(B)** levels in Rag$^{-/-}$LDLr$^{-/-}$ mice transferred with B6 (black bars) or B6.*Sle1.2.3* (grey bars) CD4$^+$ T cells. In both panels, n= 6-8 mice per group.

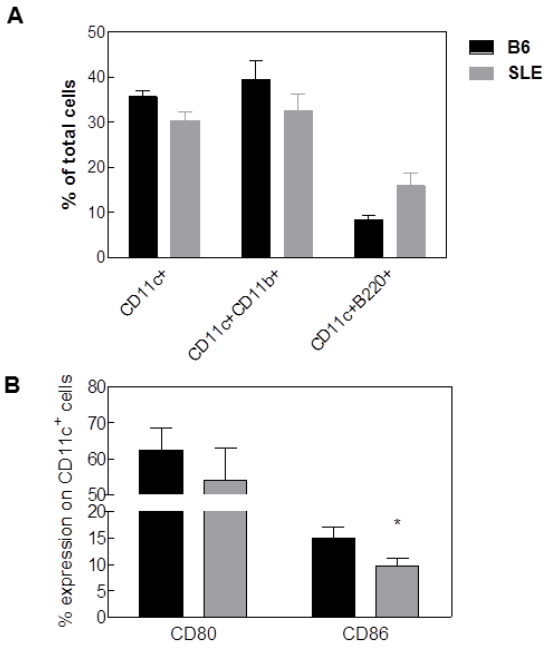

Figure 25. Analysis of antigen presenting cells in Rag$^{-/-}$LDLr$^{-/-}$ mice at sacrifice. (A) Percentages of various antigen presenting cell populations in Rag$^{-/-}$LDLr$^{-/-}$ mice transferred with B6 (black bars) or B6.*Sle1.2.3* (grey bars) CD4$^+$ T cells. **(B)** Percentage of CD11c$^+$ cells expressing CD80 (right panel) or CD86 (left panel) in Rag$^{-/-}$LDLr$^{-/-}$ mice transferred with B6 (black bars) or B6.*Sle1.2.3* (grey bars) CD4$^+$ T cells. N=3 mice per group. Experiment repeated at least twice. * indicates a *p* value < 0.05 compared to control mice.

Discussion

It is well known that T cells contribute to the pathogenesis of both SLE and atherosclerosis. Yet, our results represent the novel finding that *Sle1.2.3* expression on T cells alone is enough to enhance atherosclerosis. Indeed this is significant as it indicates that T cells play an indispensable role in SACVD.

In our study we found that transfer of B6.*Sle1.2.3* T cells to $Rag^{-/-}LDLr^{-/-}$ mice not only led to increased atherosclerosis but also led to increased T cell accumulation in the lesion. These results have several implications. Firstly, they show that Sle expression on antigen presenting cells, such as macrophages or dendritic cells, is necessary to sustain T cell hyperactivity. We know that before transfer, B6.*Sle1.2.3* T cells were hyperactive as there was increased CD25 expression (Figure 22B). However at the end of our adoptive transfer studies we found no differences in activation marker expression between B6 and B6.*Sle1.2.3* T cells. Zhu *et. al*. (150) has reported that expression of *Sle3* on antigen presenting cells mediates T cell hyperactivation in B6.*Sle3* mice. We believe that our results also validate their conclusions.

T cell trafficking to sights of inflammation is mediated by a number of receptors and adhesion molecules. These include Very Late Antigen 4 (VLA4), CD44, Signaling Lymphocyte Activation Molecule (SLAM) family receptors, and selectins (177, 178). It is known through genetic dissection that *Sle1* contains genes that encode for SLAMF6 along with L-, P- and E-selectin (37). In SLE patients, SLAMF6 expression was shown to promote Th17 cell differentiation (179). Taking into account these data, further investigation of the molecular mechanisms controlling T cell migration are merited and we hypothesize that changes in these markers are important for SACVD progression.

In assessing the phenotype of transferred T cells we observed a two fold increase in the percentage of B6.*Sle1.2.3* expressing the regulatory markers CD25 and

Foxp3. This is unusual as previous studies characterizing this model found that these mice had decreased regulatory T cells (117). Regulatory T cells are generally thought to be anti-atherosclerotic and protective against SLE yet here we find that the B6.*Sle1.2.3* Treg population transferred to $Rag^{-/-}LDLr^{-/-}$ mice was unable to protect against SACVD. This is congruent with previous studies from others (82, 117) and unpublished observations from our laboratory which show that regulatory T cell functions are perturbed in B6.*Sle1.2.3* mice; and indicates that in SACVD, restoring Treg functions may be advantageous in inhibiting disease pathogenesis.

Finally, we show that modulation of T cell function through MMF treatment can be an effective treatment in SACVD. While the implications of these data are discussed in (53), the data from the published study were included in this chapter to re-emphasize the fact that T cells play a central role in SACVD.

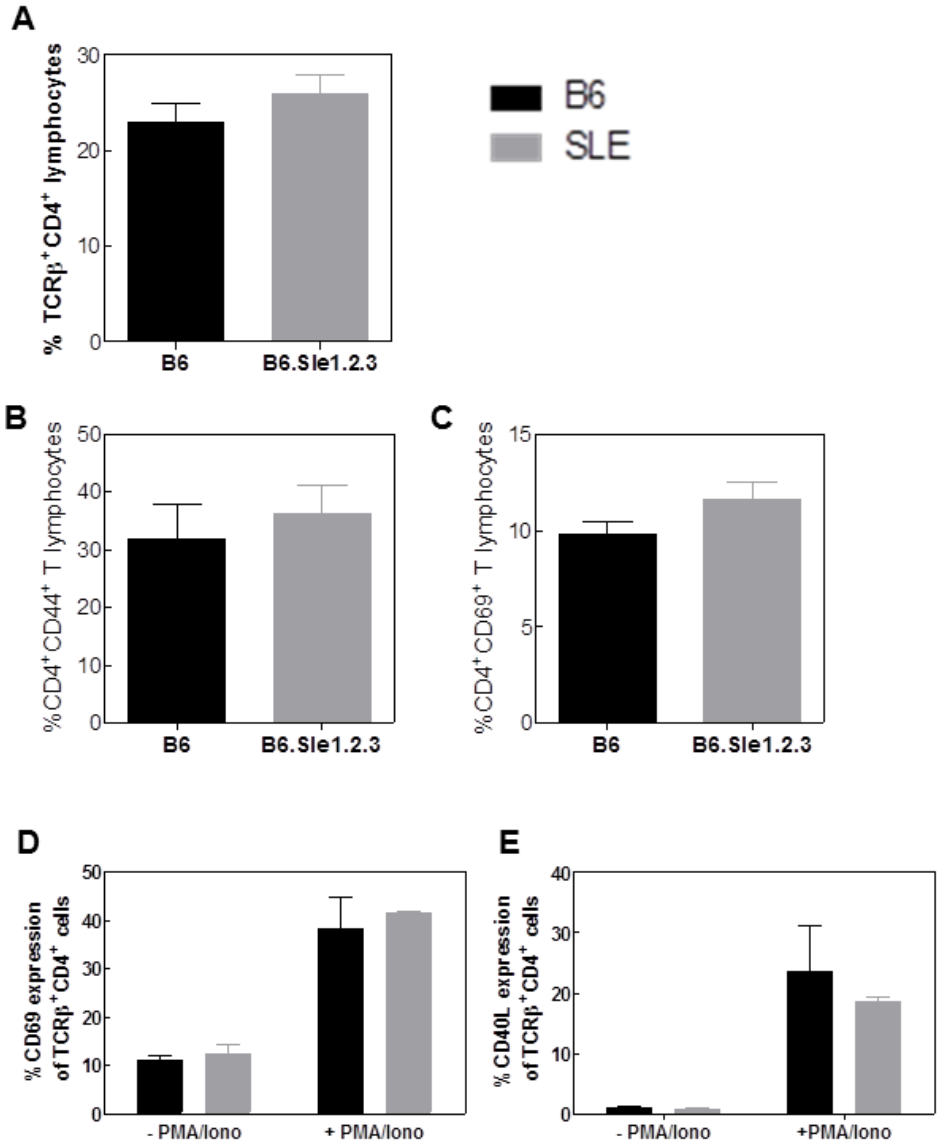

Figure 26. Analysis of T cell populations cells in Rag$^{-/-}$LDLr$^{-/-}$ mice at sacrifice. (A) Percentage of splenic CD4$^+$ T cells in Rag$^{-/-}$LDLr$^{-/-}$ mice transferred with B6 or B6.*Sle1.2.3* CD4$^+$ T cells. **(B-C)** Percentage of CD4$^+$CD44$^+$ **(B)** and CD4$^+$CD69$^+$ **(C)** T cells. **(D-E)** CD69 **(D)** and CD40L **(E)** expression on TCRβ$^+$CD4$^+$ T cells with or without PMA/Ionomycin stimulation. In all panels, black bars represent B6 donors while grey bars represent B6.*Sle1.2.3* donors. N=3-5 mice per group. Experiment repeated at least twice with comparable results.

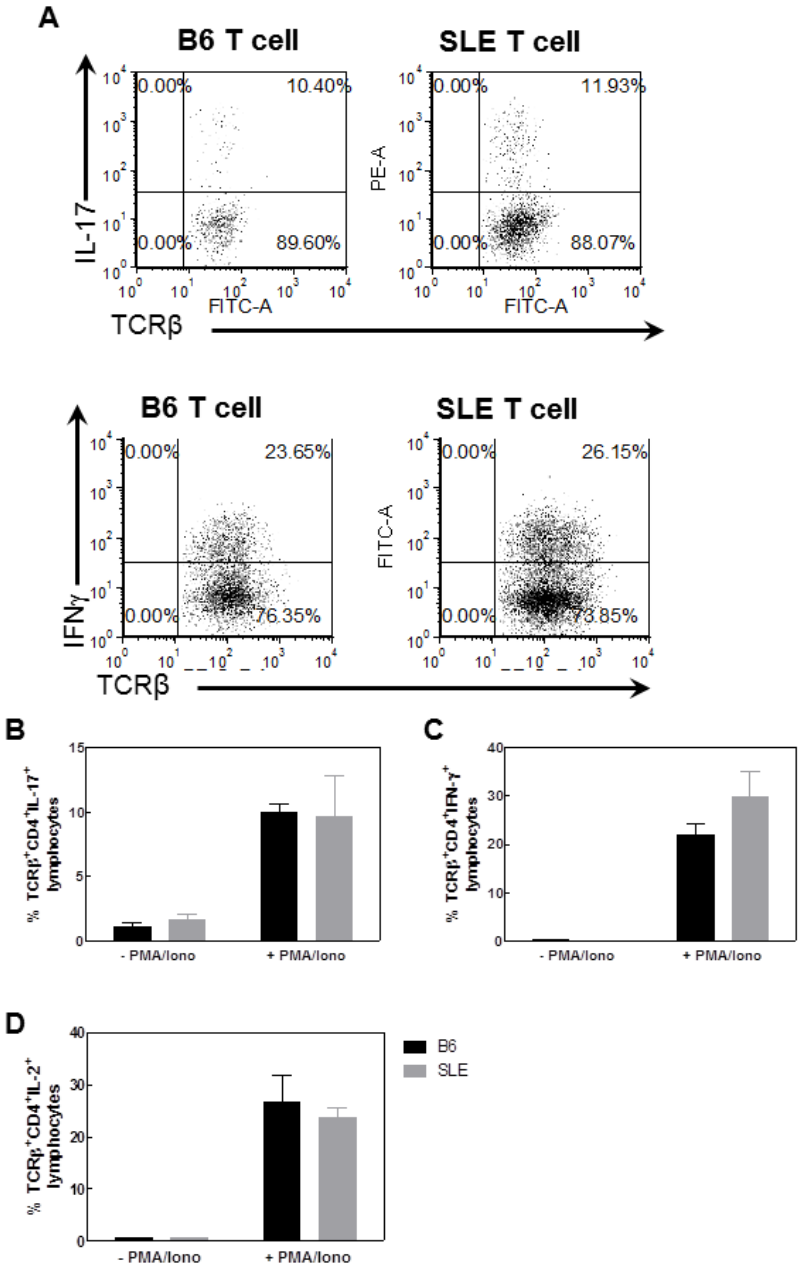

Figure 27. Analysis of intracellular cytokine expression in T cells. (A) Representative FACS plots of IL-17 expressing (top panel) and IFN-γ expressing $CD4^+$ T cells (gated on $TCR\beta^+CD4^+$ cells).**(B-D)** Percentage of $CD4^+$ T cells expressing IL-17 **(B)**, IFN-γ **(C)**, and IL-2 **(D)** after PMA/Ionomycin stimulation**.** In all bar graphs, black bars represent B6 donors while grey bars represent B6.*Sle1.2.3* donors. N=3 mice per group. Experiments repeated at least twice with comparable results.

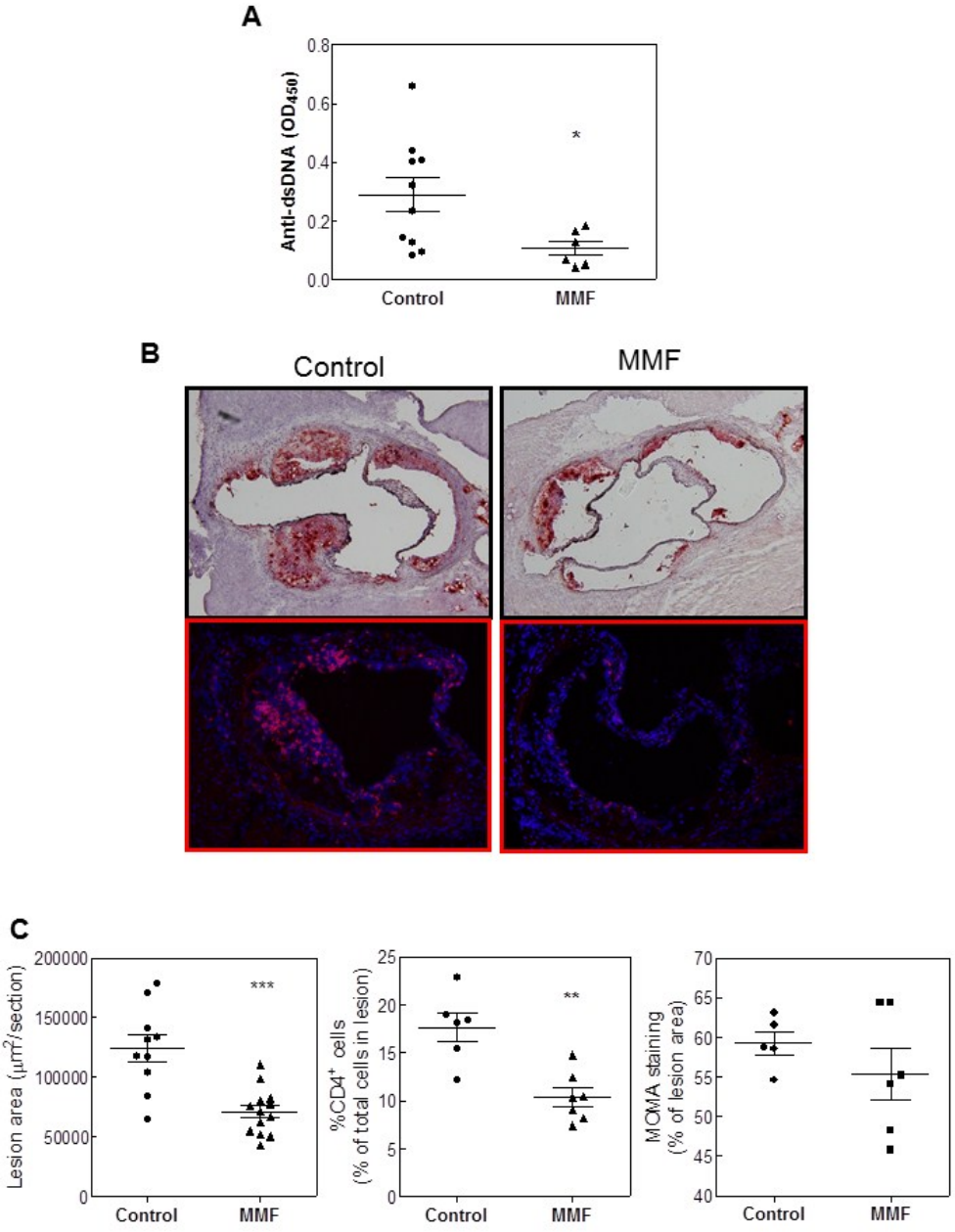

Figure 28. MMF treatment ameliorates lupus and atherosclerosis in LDLr.*Sle1.2.3* mice. (A) Serum anti-dsDNA titers in LDLr.*Sle.1.2.3* mice with or without MMF treatment. **(B)** Top panel, representative aortic sections stained with Oil-red-O. Bottom panel, representative CD4 immunofluorescent staining. **(C)** Average lesion area as determined by Oil-Red-O staining (left panel). Immunohistochemical detection of $CD4^+$ T cells (middle panel) and macrophages (right panel). In all panels closed circles represent control LDLr.*Sle1.2.3* mice while closed triangles represent MMF treated LDLr.*Sle1.2.3* mice. $n \geq 6$ mice per group. * indicates a *p* value < 0.05 compared to control mice. ** indicates a p value < 0.01 compared to control mice. *** indicates a *p* value < 0.005 compared to control mice.

CHAPTER VI

The Role of Notch1 in SLE pathogenesis.

Introduction

Notch cell-surface receptors are some of the most evolutionarily conserved proteins that function in cell fate determination. In mammals, there are four Notch proteins (Notch1-Notch4) and five canonical Notch ligands, Delta-like 1 (Dll1), Delta-like 3 (Dll3), Delta-like 4 (Dll4), Jagged1 and Jagged2. Upon ligand interaction, Notch undergoes two consecutive cleavages. The first is by the ADAM protease TACE which cleaves the extracellular portion; subsequently gamma secretase cleaves the transmembrane domain. This results in an intracellular form of Notch that translocates to the nucleus where it associates with CSL (RBP-J in humans) and other transcriptional activators to regulate the transcription of various genes such as *hes1* and the *deltex* family genes (180, 181).

The role of Notch signaling in lymphoid development has been well characterized. In the absence of Notch1, thymic lymphoid progenitors fail to initiate T cell development and instead adopt a B cell fate (180, 182). Conversely, overexpression of Notch1 in bone marrow progenitor cells inhibits B cell development (183). While the role for Notch in T cell development is known, less is known about the role of Notch in peripheral T cell regulation. Several studies have suggested that specific Notch-ligand interactions can mediate T cell polarization. For example, Dll1-Notch3 interaction is thought to direct $CD4^+$ T cells toward a Th1 cell fate (184) while Jagged1-Notch interaction leads to a Th2 cell fate (185, 186). Recent reports suggest that Notch1 may also play a role in Treg versus Th17 cell differentiation (187). Notch-Dll4 interaction

mediated by TLR9 expression was found to enhance Th17 responses in murine mycobacterium infection (188). A more recent study showed that dll4 blockade promotes Treg development (189). Additionally, Hall and colleagues found that TLR9 signaling limits iTreg conversion both *in vitro* and in an *in vivo* model of inflammatory bowel disease (190). These studies indicate that the pathogenesis of autoimmune disease could be mediated in part by dysregulation in Notch1-mediatedTh17/Treg fate decision.

Recently, there have been a number of studies implying a role for Notch in mediating autoimmune diseases. A study by Samon *et. al*. (191) found that mice carrying a Notch1 antisense transgene, which causes a 20-40% reduction in Notch1 levels, developed an autoimmune phenotype characterized by hepatic lymphocyte infiltration and reduced peripheral regulatory T cell expression. Pharmacological inhibition of Notch was found to attenuate symptoms of experimental autoimmune encephalomyelitis (EAE). Additionally, another study found that T cells from MRL-*lpr* mice have increased Notch1 levels and that inhibition of Notch1 decreases autoimmunity and nephritis (192). Give these findings, our lab sought to determine if Notch1 expression is dysregulated in our autoimmune B6.*Sle1.2.3* mice and if *in vivo* Notch inhibition in our mouse model could affect SLE disease. We hypothesized that SLE mice have increased Notch1 expression and that this increase in Notch1 expression 1) is due to increased Dll4 expression on antigen presenting cells and 2) promotes Th17 differentiation in a TLR9-dependepent mechanism (see Figure 29 for proposed mechanism). We also hypothesized that Notch inhibition would ameliorate disease progression in B6.*Sle1.2.3* mice with active SLE. Our results show that *in vivo* Notch1 inhibition ameliorates SLE pathogenesis; however, the increased Th17 cell differentiation that we observe occurs in a Notch-TLR9 independent manner.

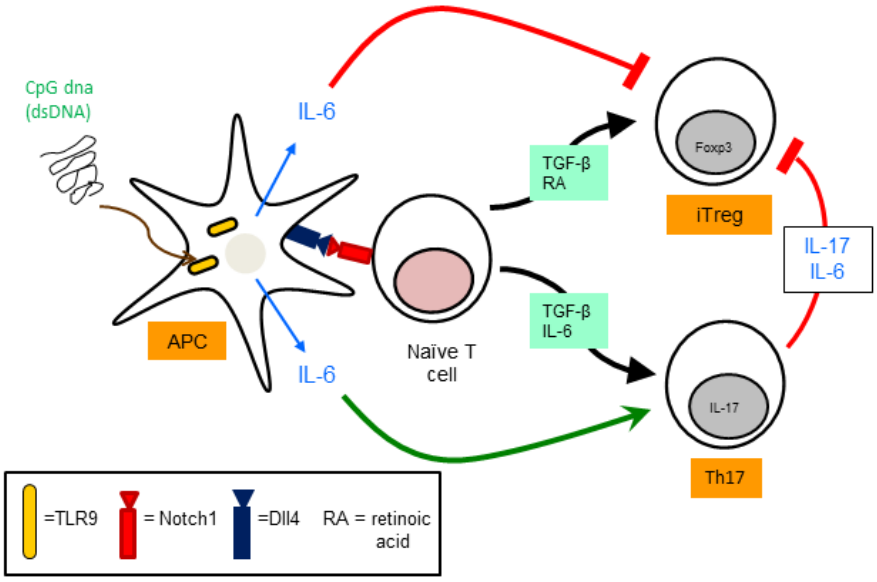

Figure 29. Schematic diagram of hypothesized pathway of Th17 development. CpG DNA stimulates TLR9 activation which upregulates Dll4 expression on antigen presenting cells. Notch1-Dll4 interaction in combination with IL-6 secretion by antigen presenting cells promotes Th17 differentiation and inhibits iTreg differentiation.

Materials and Methods

Mice. C57Bl/6 (B6) mice were originally obtained from The Jackson Laboratory and were maintained in our colony. The lupus congenic B6.*Sle1.2.3* strain has been described and characterized extensively (37-39, 105-108). All mice were maintained and used according to the guidelines and the approval of the Vanderbilt University Institutional Animal Care and Use Committee.

***In vivo* Notch inhibition.** To inhibit Notch *in vivo*, B6.*Sle1.2.3* mice were fed a chow diet containing LY 411575 ad libitum. The LY 411575 diet is a Harlan-Teklad mouse/rat chow formulated with a combination of 2 enantiomers of the gamma secretase inhibitor LY 411575, used at a ratio of 80%:20%, respectively. It delivers 5 mg/kg per day. Serum was collected every 4 weeks to determine disease progression by ELISA and body

weight was recorded weekly. At 18 weeks mice were sacrificed, organs removed and analyzed for the severity of SLE.

Western blotting. Cell lysates were loaded on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked in 3% milk/PBS-T, and incubated with the primary antibody for one hour. This was followed by incubation with an anti-rabbit or anti-mouse IgG-HRP (Promega, Madison, WI) secondary for 45 minutes and blot development by ECL reagent. Primary antibodies: mouse anti-Notch1 (eBioscience), rabbit anti-mouse dll4 (AbCam).

Cytokine ELISAs. Supernatant cytokine levels were measured by sandwich ELISA according to the manufacturer's protocol (BD Pharmingen).

Serum Enzyme-Linked Immunosorbent Assays (ELISAs). Serum titers of dsDNA were measured according to the method of Shivakumar et al. (152). Anti-oxLDL antibodies were measured as described previously (151). Serum antibody titers against β2-glycoprotein I (β2GP1) were measured by coating a 96-well Maxisorb plate with 10 μg/ml of purified β2GP1 in 1% bovine serum albumin (1% BSA)/PBS overnight. Plates were blocked in 1% BSA/PBS for two hours at room temperature. Mouse serum was added at a dilution between 1:500 and 1:5000 and incubated overnight at 4°C. Plates were washed with 0.5% Tween-20/PBS (PBS-T) and incubated with biotin-conjugated goat anti-mouse Ig(H+L) (SouthernBiotech, Birmingham, AL) for 45 minutes at room temperature then incubated with avidin-peroxidase for 30 minutes at room temperature. Plates were then washed with PBS-T and developed using TMB substrate (BD Bioscience). Cardiolipin antibody titers were determined by coating a 96-well Maxisorb plate with cardiolipin (Sigma-Aldrich) (15μg/ml in 95% ethanol). The cardiolipin ELISA was then conducted as described above for β2GP1.

Flow Cytometry. For flow cytometric analyses, spleens were removed and processed through a 0.70 μm mesh screen. Cells were counted, resuspended in 1% bovine serum

albumin (BSA) in HBSS with 0.02% sodium azide and 0.035% sodium bicarbonate, and incubated with appropriate antibodies for 40 minutes at 4°C. Cells were then washed and analyzed using a 7-laser MACSQuant Analyzer flow cytometer and FCS Express software (De Novo Software).

Intracellular cytokine staining. For intracellular cytokine staining, splenocytes were stimulated with 10ng/ml phorbol myristate acid (PMA) and ionomycin (1μg/mL) in the presence of GolgiStop or GolgiPlug for 4-5 hous at 37 °C and 5% CO_2. Cells were then stained for extracellular antibodies, washed, and fixed overnight in 2% paraformaldehyde in PBS. Cells were then permeabilized with Cytoperm (BD Bisciences) reagent, stained with the appropriate intracellular antibodies and analyzed by flow cytometry. For IL-17 secretion, instead of incubating splenocytes for 5 hours in the presence of GolgiPlug, cells were first incubated with PMA and ionomycin without Golg-Plug for two hours then incubated and additional three hours in the presence of GolgiPlug.

Statistical Analyses. Statistical analyses were conducted using PRISM 5.0 software (GraphPad Software Inc., La Jolla, CA). A Student's t-test was used to calculate significant differences between groups. A p value < 0.05 was considered significant.

Results

Notch1 expression is upregulated in older SLE mice.

In order to assess activated Notch expression levels, we isolated $CD4^+$ T cells from spleens of B6 and B6.*Sle1.2.3* mice and stimulated them with plate bound anti-CD3 and anti-CD28 for 48 hours *in vitro*. After stimulation, we assessed intracellular Notch1 ($Notch1^{IC}$) (indicative of active Notch) levels through western blotting. We found that while there was no difference in $Notch^{IC}$ expression between younger B6 and

B6.*Sle1.2.3* mice (Figure 30, left panel), older B6.*Sle1.2.3* mice had increased Notch1^{IC} protein expression (Figure 30, right panel). These data suggest that Notch1 is dysregulated in B6.*Sle1.2.3* mice, and that this dysregulation correlates with disease progression.

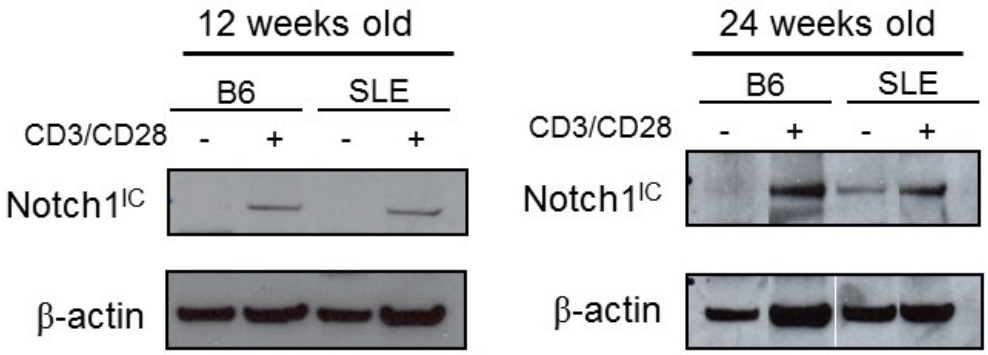

Figure 30. Notch activity correlates with disease progression in B6.*Sle1.2.3* mice. Notch1^{IC} expression in isolated CD4+ T cells from mice at 8 weeks **(A)** and 28 weeks **(B)** of age. CD4^{+} T cells were isolated and cultured for 48 hours and lysates were analyzed for Notch1^{IC} and β-actin expression by Western blot.

***In vitro* Notch1 inhibition decreases serum lipoprotein levels and autoantibody production B6.*Sle1.2.3* mice.**

Two separate papers have implicated a role for Notch1 in autoimmunity. Samon *et al.* (191) showed that *in vivo* inhibition of Notch1 through gamma secretase inhibition in B6 mice led to autoimmune hepatic lymphocyte infiltration. Additionally, Teachy *et al.* showed that gamma secretase treatment in autoimmune MRL-*lpr* mice ameliorated autoimmune disease progression by decreasing the double negative T cell population in lymphoid organs; this led to decreased dsDNA antibody titers, decreased splenomegaly and lympho-proliferation (191, 192). Given these two disparate reports, our laboratory

sought to determine if *in vivo* Notch inhibition in our mouse model could affect SLE disease.

Eight week old B6.*Sle1.2.3* mice were fed a diet containing gamma secretase inhibitor (GSI) (LY411575) or control diet for 18 weeks. Consistent with other studies (193) GSI diet feeding led to a progressive greying of fur (Figure 31A), indicative of Notch inhibition. Upon further examination, these experimental mice exhibited splenomegaly and decreased body weight (Figure 31B-C). The decrease in body weight was not due to decreased food intake as both groups had similar weekly food intake levels (Figure 31D).

To confirm that GSI treatment led to Notch inhibition, splenocytes were harvested and stimulated with anti-CD3/anti-CD28 for 72 hours. Western blotting of cell lysates was performed to assess $Notch^{IC}$ protein levels. As shown in Figure 31E-F, upregulation of $Notch^{IC}$ upon stimulation is impaired in GSI diet fed mice thus confirming inhibited Notch activity.

Notch inhibition decreases autoantibody production and serum cholesterol levels.

In order to determine the effect of Notch inhibition, serum was collected every four weeks and autoantibody titers measured. At 12 weeks of diet, there were no differences in autoantibody titers between the two groups, with anti-dsDNA titers steadily rising in both groups. However, at sacrifice (18 weeks on diet) GSI-fed B6.*Sle1.2.3* mice had decreased serum antibody titers against dsDNA, β2GP1, and cardiolipin. (Figure 32A-C)

Serum lipoprotein analyses revealed that mice on GSI had decreased serum cholesterol levels with no difference in serum triglyceride levels (Figure 32D-E). The decrease in serum cholesterol was found to be due to a decrease in the non-VLDL fractions (Figure 32F).

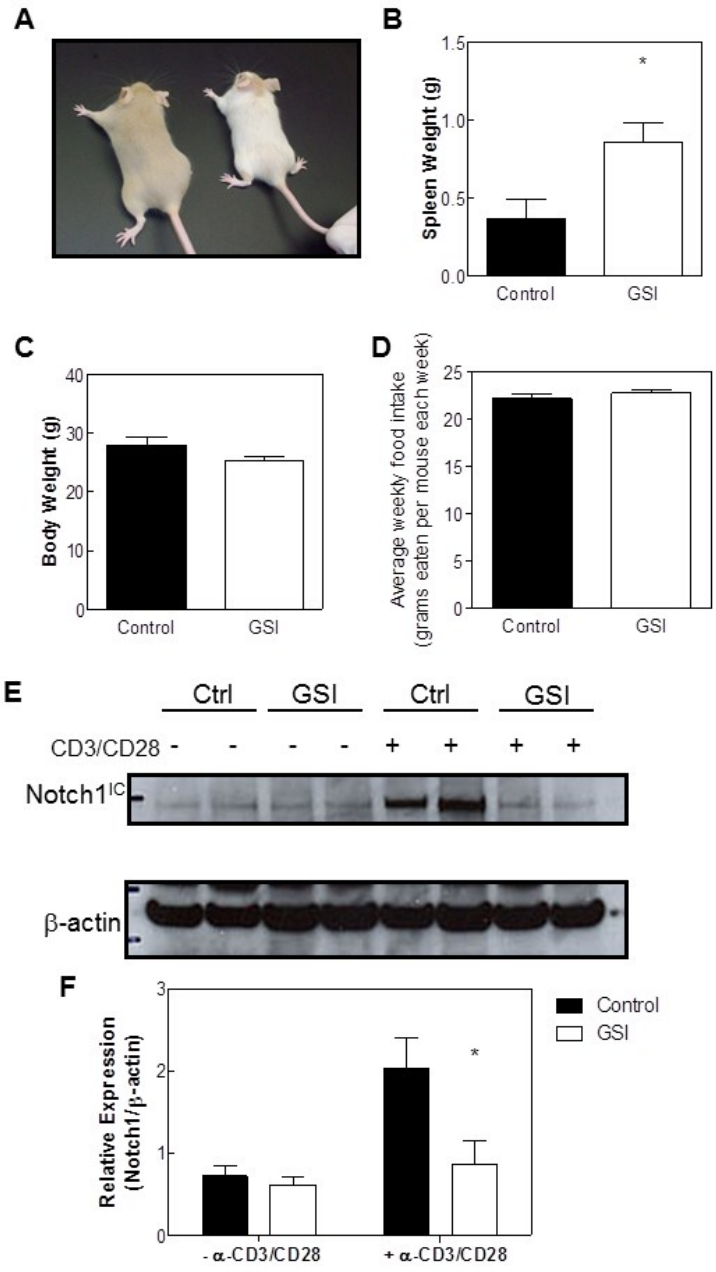

Figure 31. Effects of *in vivo* Notch inhibition. (A) Evidence of progressive hair greying in GSI-fed mice (on right) but not control diet-fed mice (on left). **(B)** Average spleen weight in control diet-fed and GSI-fed B6.*Sle1.2.3* mice. **(C)** Average body weight in control diet-fed and GSI-fed B6.*Sle1.2.3* mice. **(D)** Average weekly food intake (per mouse, per week) in control diet-fed and GSI-fed mice. N=3-4 mice per group.**(E)** Confirmation of *in vivo* inhibition of Notch activity in GSI-fed but not control-fed B6.*Sle1.2.3* mice. Splenocytes were harvested from mice and cultured for 72 hours with or without αCD3/αCD28 stimulation. Whole-cell lysates were prepared and $Notch1^{IC}$ and β-actin expression was detected by Western blot. Data are representative of n = 3 (control) and n = 4 (GSI). **(F)** Graphic representation of band intensities from panel **E**. Expression of $Notch1^{IC}$ was normalized to β-actin expression. * indicates a p value < 0.05 compared to control fed mice.

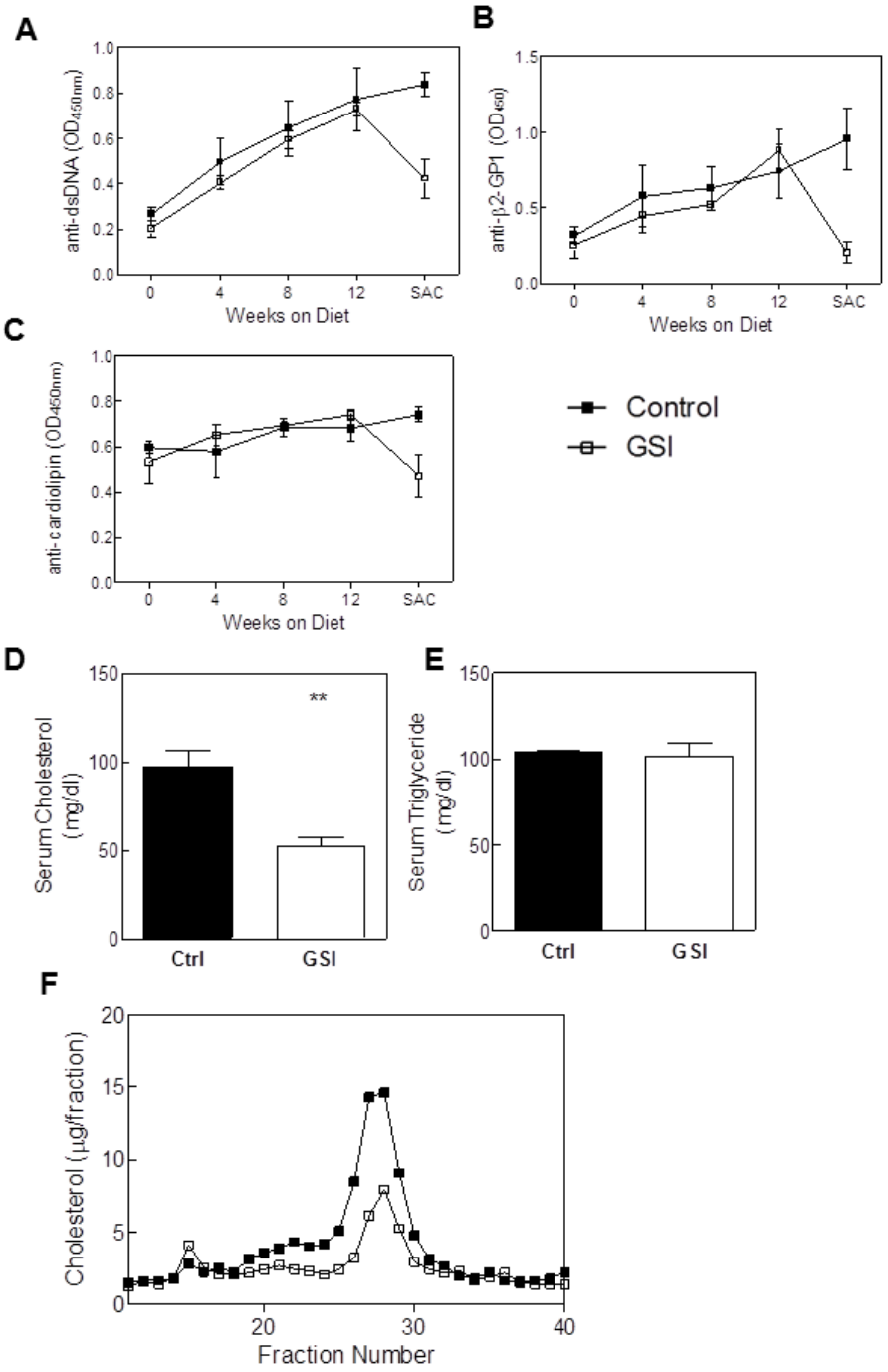

Figure 32. Effects of Notch inhibition on auto-antibody titers and serum lipoprotein levels. (A-C) Serum antibody titers of anti-dsDNA (**A**), anti-β2GP1 **(B)**, and anti-cardiolipin **(C)** antibodies in B6.*Sle1.2.3* mice fed control (closed bars) or GSI (open bars) diet. **(D-E)** Serum cholesterol **(D)** and triglyceride **(E)** levels in B6.*Sle1.2.3* mice fed control (closed bars) or GSI (open bars) diet. In A-E, n=3-4 mice per group. **(F)** FPLC analysis of cholesterol lipoprotein distribution B6.*Sle1.2.3* mice fed control (closed bars) or GSI (open bars). Serum from 3-4 mice per group was pooled. ** indicates a p value < 0.01 compared to control fed mice.

Effects of Notch inhibition on immune cells.

As shown before, GSI diet feeding led to splenomegaly in B6.*Sle1.2.3* mice. Flow cytometric analyses showed that this increase in spleen weight was due to an increase in $CD4^+$ T cells and $CD11b^+$ macrophages (Figure 33A). We also found a decrease in the percentage of $CD19^+$ B cells, partly explaining the decrease in autoantibody production (Figure 33B), and a slight increase in regulatory T cells (Figure 33C).

TLR9 stimulation does not upregulate cytokines associated with Th17 differentiation in B6.*Sle1.2.3* mice.

In our proposed model, increased Dll4 expression on antigen presenting cells, resulting from increased in Notch expression mediates enhanced TLR9-CpG stimulation. To determine if increased Notch1 expression is due to increased Dll4 expression on antigen presenting cells in B6.*Sle1.2.3* mice $CD11c^+$ (dendritic cells) and $CD19^+$ (B cells) were isolated by magnetic separation from splenocytes of B6 and B6.*Sle1.2.3* mice.

$CD11c^+$ and $CD19^+$ cell populations were stimulated with the TLR9 agonist CpG DNA. After stimulation, Dll4 protein expression was analyzed by western blot. We found that after stimulation, there were no differences in Dll4 expression levels on dendritic cells (Figure 34A) or B cells (not shown) in neither control nor B6.*Sle.1.2.3* mice. We also harvested supernatants from these stimulations and assessed IL-6, IL-1β and IL-23 secretion levels. While we could not detect IL-1β or IL-23, we found a significant decrease in IL-6 levels from B6.*Sle1.2.3* mice compared to control (Figure 34B), indicating that CpG stimulation does not facilitate the increased Th17 differentiation that we observe in these mice.

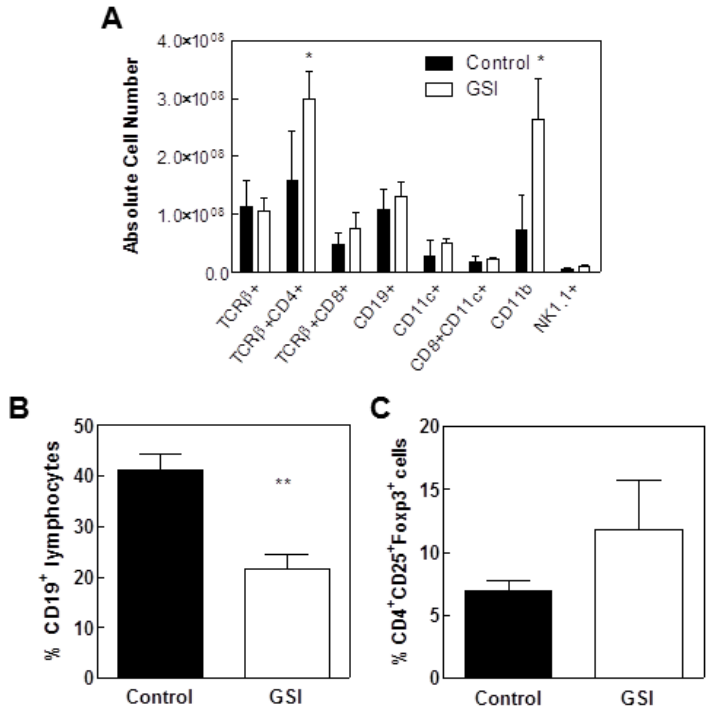

Figure 33. Analysis of splenocyte populations in GSI-fed B6.*Sle1.2.3* mice. (A) Absolute numbers of spleen cell populations in B6.*Sle1.2.3* mice fed control (open bars) or GSI (control bars) diet. **(B-C)** Percentage of B lymphocytes **(B)** and regulatory T cells **(C)** in B6.*Sle1.2.3* mice fed control (closed bars) or GSI (open bars) diet. In all panels, n=3-4 mice per group. * indicates a *p* value < 0.05 compared to control fed mice. ** indicates a p value < 0.01 compared to control mice.

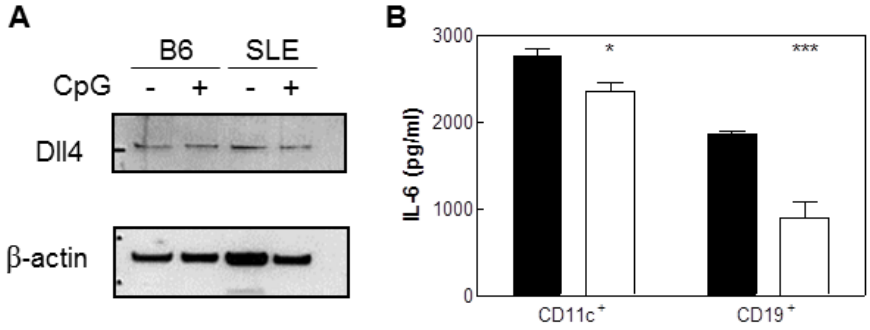

Figure 34. CpG stimulation on does not upregulate dll4 expression or IL-6 secretion in B6.*Sle1.2.3* mice. (A) Dll4 expression by $CD11c^+$ splenocytes from B6 and B6.*Sle1.2.3* mice. Splenocytes were harvested from mice and $CD11c^+$ cells isolated by magnetic separation. Isolated cells were cultured with CpG-DNA for 48 hours and lysates prepared. Dll4 and β-actin expression was detected by Western blot. Data are representative of 4 mice per group. **(B)** IL-6 secretion by $CD11c^+$ and $CD19^+$ cells stimulated for 48 hours with CpG-DNA. Closed bars represent B6 mice while open bars represent B6.*Sle.1.2.3* mice. N=3-4 mice per group. ** indicates a p value < 0.01 compared to control mice. *** indicates a p value < 0.001 compared to control mice.

Discussion

In this chapter we investigated how Notch inhibition affects SLE pathogenesis. We found that while inhibiting Notch activity delayed SLE onset, this delay was not through the mechanism we originally hypothesized. Although we saw a decrease in autoantibody and phospholipid antibody titers, there was no increase the percentage of regulatory T cells after treatment. Additionally, we found that TLR9 activation of antigen presenting cells, through CpG DNA stimulation, did not increase IL-6 secretion by these cells. These results lead us to believe that Notch modulation of SLE pathogenesis may function in an alternate, TLR9-independent manner.

Notch1 and its signaling molecules are expressed on many cell types including T cells, B cells and macrophages. A number of studies have found that macrophage activation by various TLR ligands can lead to Notch upregulation on macrophages (194, 195). Moreover, it has been shown that GSI treatment in an activated lymphocyte-derived DNA (ALD-DNA) immunization model of SLE results in amelioration of SLE symptoms by inhibiting macrophage activation and M2b cell polarization (196). Given these studies it is possible that GSI treatment led to the alteration of other cell types which led to the amelioration of SLE disease. Additionally, it is possible that activation of other TLR ligands and or Notch1 ligands could lead to Th17 cell differentiation. These and other options remain to be explored in our model.

Similar to one study (192), we found that GSI treatment led to a decrease in $CD4^+$ T cells (Figure 34A) and double negative T cells (data not shown). Yet, in our study, GSI treatment was also associated with splenomegaly and decreased body weight, which was not previously observed. There are several potential explanations for these discrepancies. The first is the type of GSI inhibitor used. In Teachey *et al*. N-S-phenyl-glycine-t-butyl ester (DAPT) was given by gavage five days a week at a 5mg/kg dosage. The authors chose this drug and dosage scheme to limit the amount of drug

toxicity. In our studies we fed the mice a diet with LY411575 ad libitum, which delivered 5mg/kg of the drug per day. Toxicities associated with GSI inhibition include thymic atrophy and increased intestinal goblet cell formation (197). Upon gross examination of our mice we found some of these morphologies indicating the possible existence of drug toxicity. Additionally, while our study was a preventative study, the study referenced above was a drug efficacy study. Their treatment didn't start until the mice were five or six months old, an age at which SLE symptoms are at their peak, while our studies began in 2 month old mice in order to delay disease onset. Finally, while we used our triple congenic B6.*Sle1.2.3* disease model, they used the Mrl/lpr model which is mainly a model of lymphoproliferative disease. Though there were several differences between the two studies, inhibition of SLE was the end result of both studies suggesting to us that GSI treatment could be an attractive therapy for the treatment of SLE.

CHAPTER VII

GENERAL DISCUSSION

Summary and Proposed Mechanism.

In this body of work, we have shown that SLE and atherosclerosis susceptible LDLr.*Sle1.2.3* mice have increased atherosclerosis and that $CD4^+$ T cells play a significant role in mediating this co-morbidity. In our initial studies we find that SLE accelerates atherosclerosis and that this enhanced atherogenesis is independent of dietary lipids. Additionally, we observe that increased T cell accumulation occurs in the lesions of LDLr.*Sle1.2*.3 mice and that T cells from SLE mice display dysregulated phenotypes commonly observed in SLE patients. Next, we show that transfer of individual lupus susceptibility loci associated with T cell hyperactivity is not sufficient to cause accelerated atherosclerosis; however, they mediate pathologies associated with lupus. Finally we demonstrate that while individual loci aren't able to exacerbate atherosclerosis in SLE m ice, transfer of $CD4^+$ T cells that contain all three loci into immunodeficient mice can enhance atherogenesis, implying that B6.*Sle1.2.3*. T cells alone are sufficient to induce SACVD.

In addition to demonstrating that T cells play an critical role in SACVD, our results show that the mode by which T cells regulate this enhanced disease is multifaceted. Conventional T effector cells in SLE mice have a pro-inflammatory, pro-migratory phenotype which favors T cell infiltration into the atherosclerotic lesion. This increased T cell accumulation in the lesion is the primary mode of enhanced atherogenesis. Additionally, there is a defect in the differentiation of anti-inflammatory, anti-atherogenic regulatory T cells which augments the disease process. These studies

ultimately show that modulation of T cell function could be an attractive therapeutic target in treating at risk SLE patients.

Figure 35, illustrates our proposed mechanism of SACVD. We believe that conventional T cell accumulation within the plaque drives the atherosclerotic process in SLE-susceptible mice. Correspondingly, impaired Treg development and function allows for increased T cell activation, lesional T cell accumulation, and systemic inflammation.

Regulation of SACVD by conventional T cells.

Conventional T cells are known to have a largely pro-atherogenic role in traditional atherosclerosis. Both human and animal studies have revealed that the T cells found in lesions are largely $CD4^+$ and pro-inflammatory (198). In our adoptive transfer studies (**Chapter V**) we found that transfer of B6.*Sle1.2.3* T cells from diseased mice led to increased atherosclerosis compared to mice with B6 T cells. Additionally, while B6.*Sle1.2.3* T cells were hyperactive before transfer, there were no differences in the activation status of either group at sacrifice. This suggests that B6.*Sle1.2.3* T cells possess a hyper-migratory phenotype. However, our studies did not address whether the increased lesional T cell accumulation is a consequence of hyperactivation before transfer (due to their micro-environment) or if these two phenotypes are distinct. A simple experiment to test this hypothesis would be to repeat the adoptive transfer experiment, adding a group in which B6 T cells are activated *in vitro* prior to being transferred to $Rag^{-/-}LDLr^{-/-}$ mice. The results from this study will tell us if T cell activation is solely responsible for increased T cell infiltration into the plaque. Furthermore, we can also determine if B6.*Sle1.2.3* T cells from younger mice (pre-disease onsent) would have the same effect as older B6.*Sle1.2.3* T cells (nature versus nurture).

Our results may also indicate that while B6.*Sle1.2.3* T cells are efficient in traveling to the atherosclerotic plaque, they may be unable to emigrate out of the lesion

to secondary lymphoid organs. As a result, their inability to emigrate leads to increased T cell accumulation. Galkina *et al.* (199) observed that leukocyte trafficking into the aorta is partially dependent on CD62L in ApoE$^{-/-}$ mice. Additionally, two groups have found that the chemokine receptor CCR7 is important for the emigration of T cells from the atherosclerotic lesion; whereas absence of this receptor leads to lymphocyte accumulation and inhibits cell emigration in atherosclerosis-prone mice (200, 201). Given these published studies and our observations, the examination of these proteins and others involved in T cell migration will offer great insight into how T cell trafficking into/out of the lesion is regulated and its effects on SACVD progression.

Regulation of SACVD by regulatory T cells.

In addition to dysregulated conventional T effector cells, B6.*Sle1.2.3* mice also have impaired Treg development and function. We found that B6.*Sle1.2.3* mice have either the same or slightly elevated percentages of Tregs, contrasting previously published data by Cuda *et. al.*(117). Nonetheless, our own unpublished *in vitro* observations have indicated that these B6.*Sle1.2.3* Tregs are dysfunctional an unable to suppress conventional T cell functions mirroring data from the above studies. Furthermore, we have shown that Treg development induced by TGF-β is also impaired (**Chapter II**). These observations have recently been published by the Morel laboratory using single congenic mice (202).

Studies show that Tregs have anti-inflammatory, anti-atherogenic effects (84, 85). However, therapeutic benefit of *ex vivo* expanded Tregs on traditional atherosclerosis or SACVD has not been assessed. Many studies have demonstrated that transfer of iTregs can prevent autoimmunity. Scalapino *et al*. (146) observed that transfer of iTregs expanded *ex vivo* suppressed autoimmunity and glomerulonephritis in NZB/NZW mice and Su *et al*.(145) found that iTregs protected against autoimmunity in a

SLE like model of chronic graft versus host disease. These and others indicate that expansion of Tregs may be an effective option in treating SAVD. Simple adoptive transfer techniques using *ex vivo* expanded iTregs can be used to determine if iTregs can inhibit progression of SACVD.

In **Chapter VI**, we assess the role of the Notch1, a protein commonly studied in cell fate determination, in Treg/Th17 homeostasis. Although, we find that *in vivo* inhibition of Notch1 does not directly alter the Treg/Th17 balance, there have been a number of recent studies to explore other methods of reestablishing this balance. In a review by Yang *et al*. (203) four therapeutic agents that restore immune balance between Tregs and Th17 cells are discussed; these include all-trans retinoic acid (ATRA), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), rapamycin and H471-94 (a nucleosomal histone peptide epitope). While ATRA maintained Foxp3 expression, rapamycin was more effective in expanding human nTregs *ex vivo*; and their combined use increased the suppressive activity of expanded Tregs (204). Future studies in our laboratory are aimed at determining if treatment of LDLr.*Sle1.2.3* with these agents could positively regulate SACVD pathogenesis.

Observations from LDLr.*Sle1.2.3* mice.

While this dissertation focuses on the role of T cells in SACVD, it is important for us to highlight and discuss observations from our studies not necessarily related to T cells. In the majority of our radiation chimera studies we found that mice containing lupus susceptibility loci had decreased serum cholesterol levels compared to control mice at sacrifice. There was no correlation between cholesterol levels and atherosclerotic lesion area, indicating that immune dysregulation and not dyslipidemia is a more important risk factor in SACVD. In addition, we found that cholesterol levels at 16 weeks post bone marrow transplant (prior to Western diet initiation) were equal between all groups as

were auto-antibody titers. These observations lead us to hypothesize that as atherosclerosis progresses, lupus-susceptible mice become more efficient in uptake and/or clearance of circulating lipoproteins, potentially through Fcγ receptor-mediated uptake of anti-oxLDL or anti-phospholipid immune complexes. Furthermore, these effects on cholesterol metabolism are not specific to any one SLE locus indicating that there is some constant between these loci which regulates cholesterol homeostasis. Elucidation of the link between cholesterol homeostasis and these lupus susceptibility loci may prove useful in designing therapies against traditional atherosclerosis, where lipid dysregulation plays a more prominent role.

In chapters 3 and 4 we found that neither lupus susceptibility loci *Sle1*, *Sle3* nor *Sle1*.3 were sufficient to accelerate atherosclerosis. This leads us to question the role of *Sle2* in mediating SACVD. *Sle2* is associated with B-1a B cell accumulation (105). On the other hand, recent studies in traditional atherosclerosis have indicated a protective role for B-1a B cells and an atherogenic role for B-2 B cells (41, 42). Xu *et.al* (205) demonstrated that combination of the *Sle2* sub-locus, Sle2c1, with *lpr* (associated with defects in fas-mediated apoptosis), leads to altered T cell homeostasis, with a skewing of T cells towards a Th17 fate and away from a Treg fate. These observations emphasize the importance of assessing the effect of *Sle2* alone on SACVD in the near future. Furthermore, one could speculate that B-1 B cell–mediated T cell differentiation/activation, through synergy with *Sle1* and/or *Sle3* could have pro-atherogenic effects, just as its synergy with *lpr* has pro-inflammatory effects. This would be an exciting finding as it would imply that SACVD and traditional CVD pathogenesis occur by two distinct mechanisms.

Finally, T cell and antigen presenting cell interactions are important for the initiation and sustentation of inflammatory processes. Dendritic cells from B6.*Sle1.2.3* mice have a hyperactive phenotype as observed in studies from the laboratories of

Wakeland and Morel (39, 107). Congruent with these studies, we found increased MHC class II (I-Ab) expression in the lesions of LDLr.*Sle1.2.3* mice (presumably on antigen presenting cells) along with increased CD86 and/or CD80 expression in our single congenic mouse studies. The hyperactive dendritic cell phenotype is dendritic cell-intrinsic and calls into question the significance T cell-dendritic cell cooperation in SLE-accelerated atherosclerosis. Indeed, it is possible that while *Sle1.2.3* T cells are sufficient to accelerate atherosclerosis, *Sle1.2.3* dendritic cells may have an additive effect and exacerbate atherosclerotic disease even more than T cells alone. This would indicate that *Sle1.2.3* expression in both T cells and DC's work cooperatively to affect vascular disease progression. Along the same lines, future studies may indicate that *Sle1.2.3* dendritic cells alone are sufficient to promote SACVD, further emphasizing the role of dendritic cells in the atherosclerotic process. The observations pointed out above accentuate the fact while T cells are important in SACVD, there are a host of other cell types/pathways waiting to be explored in both atherosclerosis and SLE.

The Role of T cells in SACVD: A Clinical Perspective

A definite correlation between autoimmunity and premature cardiovascular events in SLE patients has been established, with the finding of SLE-related CVD risk factors such as increased inflammation, increased autoantibody production and the use of certain drugs. Despite these findings, the mechanism(s) behind this co-morbidity remain elusive. It is known that perturbations of the immune system associated with traditional atherosclerosis are increased in some SLE patients; these include increased pro-inflammatory cytokine production and anti-phospholipid antibody production (45) (206). However, very few studies have provided evidence that immune dysregulations definitively promote the accelerated cardiovascular disease seen in many SLE patients.

The same is true in regards to human studies and T cells in SACVD. T cells from SLE patients are known to have signaling abnormalities which influence cell function. These abnormalities include replacement of the CD3 zeta chain with Fcγ receptor, an imbalance in transcription factor regulation and translocation, increased CD40L expression, decreased IL-2 expression and decreased activation induced cell death (77, 124, 207, 208). These aberrations are known to mediate SLE pathogenesis and thought to be important in SACVD. While one study found that decreased TGF-β activity correlated with increased LDL levels and carotid intima to media thickness (58), to date there are a very limited number of clinical studies that correlate T cell signaling abnormalities with CVD risk in SLE patients.

In LDLr.*Sle1.2.3* mice, T cells display some of the same abnormalities as discussed above in humans (see **Chapter II**). Moreover, we find that increased T cell accumulation in the atherosclerotic plaques is partly responsible for increased atherogenesis observed. We believe that data highlighted in this dissertation have broad implications in the clinical setting. The results from our studies suggest that clinical studies should now be undertaken to evaluate the connection between T cell dysfunction and cardiovascular disease risk in SLE. Relevant clinical investigations include determining if infarcts from SLE patients have more T cells present, examining the migratory capacity of T cells from SLE patients with clinical or subclinical cardiovascular disease, and undertaking correlative studies which examine the relationship between SLE associated T cell aberrations and cardiovascular disease risk factors.

Furthermore, our studies indicate that the discovery of new treatment options which target T cells should be aggressively pursued. Most current therapies for SLE target B cell function and antibody production. This is especially evident as the most recent drug approved specifically for SLE targets BAFF, a factor that promotes B cell survival and proliferation (209). Although B cells are known to be largely pathogenic in

SLE, in traditional atherosclerosis, both pro- and anti- atherogenic properties have be ascribed to B cells (41, 42, 69) attesting to the complexity of the disease process. On the other hand, the role of T cells in both SLE and atherosclerosis remains the same as they have consistently been found to be pro-inflammatory and pro-atherogenic.

Our laboratory found that treatment of LDLr.*Sle1.2.3* mice with MMF resulted in decreased lesion area which was associated with decreased lesional T cell accumulation (**Chapter V** and (53)). It is important to note that a recent study by Kiani *et al.* found that treatment of SLE patients with MMF did not improve carotid intima to media thickness or coronary artery calcification over a two year period (210). However, interpretation of the results from this study are severely limited by a number of factors including sample size, MMF dosage, CVD risk assessment parameters and time frame. Even so, the data presented in this dissertation should spur more clinical investigations that strive to understand how T cells shape the atherosclerotic process in SLE. These investigations can yield a number of discoveries—from improved clinical biomarkers to assess SACVD risk to tailored therapies for SACVD—ultimately leading to improved quality of life for SLE patients.

Concluding Remarks

In this body of work, I have taken advantage of a novel mouse model of SLE-accelerated atherosclerosis to understand the role of T cell dysregulation in SACVD. While much more remains to be discovered, our work provides confirmation that T cells are important in SACVD and that T cell intrinsic and extrinsic mechanisms facilitate this enhanced disease process. The results taken from these studies will allow us to further delve into the molecular processes that regulate SACVD, ultimately facilitating the development of therapeutics designed to treat both SLE and cardiovascular disease.

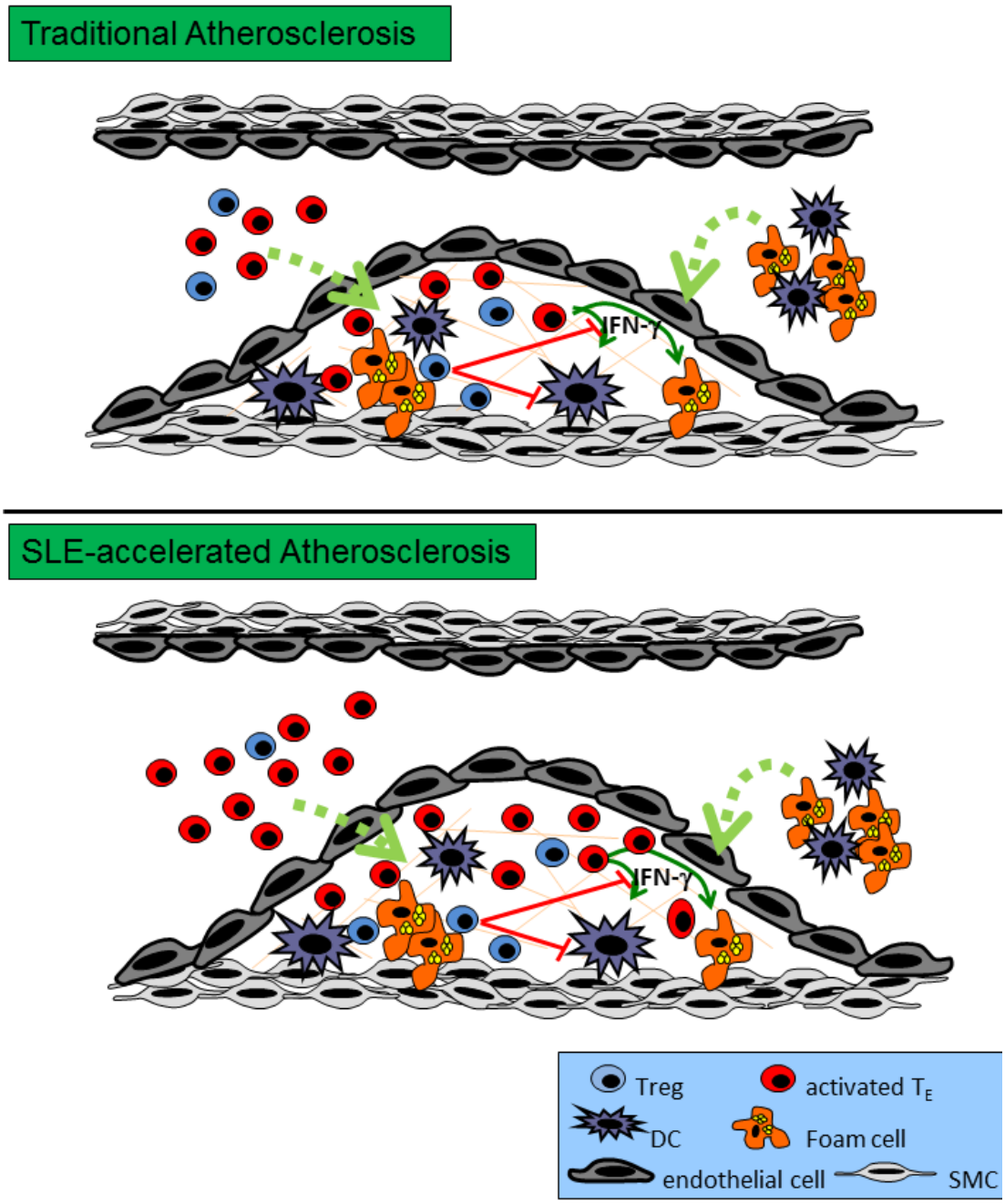

Figure 35. Proposed mechanism of SACVD. In LDLr.*Sle1.2.*3 mice, T cell accumulation within the plaque drives the atherosclerotic process. This is associated with impaired Treg development and function which allows for increased T cell activation, lesional T cell accumulation, and systemic inflammation.

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