PHENOTYPIC CHARACTERIZATION AND GENETIC DETERMINATION OF T CELL POPULATIONS ASSOCIATED WITH LUPUS SUSCEPTIBILITY LOCUS *SLE1A*

By

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To my Grandparents, my biggest fans, my unfailing support. You are both greatly missed.
"You're smart, just like your grandfather."

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Abstract of Dissertation Presented to the Graduate School
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Patients suffering from systemic lupus erythematosus (SLE) present with several manifestations, one of which being a decrease in the number of circulating regulatory T cells (Tregs). However, it has been shown that the suppressive capacity of the remaining population is maintained. SLE onset involves a combination of factors including genetic predisposition relating to the occurrence of susceptibility loci as well as environmental triggers. The goal of our lab is to locate genes involved in susceptibility by studying a congenic murine model of SLE containing three susceptibility loci from the NZM2410 spontaneous murine model bred onto a normal B6 background. The NZM2410 susceptibility loci are referred to as *Sle1*, *Sle2*, and *Sle3* corresponding to chromosomes 1, 4 and 7, and combine to confer the same phenotype as the NZM2410. Major lupus susceptibility locus *Sle1*, which comprises three independent loci, *Sle1a*, *Sle1b*, and *Sle1c*, has been shown to enhance activation levels and effector functions of CD4-expressing T cells, reduce the size of the Treg subset, which express CD4, CD25 and CD62L, as well as decrease expression of the transcription factor Foxp3 among this population preceding autoantibody production. These phenotypes can be accounted for by *Sle1a* in a T cell-intrinsic manner. Upon further analysis of *Sle1a* expression, we found that although a decrease in the Treg population was observed, these cells appeared normal in terms of expression of

markers associated with the regulatory phenotype. Both *in vivo* and *in vitro* functional studies indicated that these remaining Tregs also maintained their suppressive capacity on a per-cell basis suggesting that *Sle1a* controls the number of Tregs rather than their function. In addition, these *in vitro* and *in vivo* suppression assays showed that *Sle1a* expression induced effector T cells to be resistant to Treg suppression, as well as dendritic cells to overproduce IL-6, which inhibits Treg suppression. Overall, these results show that *Sle1a* controls both Treg number and function by multiple mechanisms, directly on the Tregs themselves and indirectly through the response of effector T cells and the regulatory role of dendritic cells. Examination of truncated regions of *Sle1a*, called *Sle1a.1* and *Sle1a.2*, resulted in intermediate phenotypes, indicating the presence of at least two genes within *Sle1a* synergistically interacting to confer the observed increase in activation of CD4-expressing T cells and decrease in Treg population.

According to Ensembl Release 40 (www.ensembl.org), only one gene is present in the *Sle1a.1* region of murine chromosome 1, which encodes for the protein pre-B cell leukemia transcription factor 1 (Pbx1). Little is known about the role of Pbx1 in immune cells and only two isoforms have been previously described, Pbx1-a and Pbx1-b. We found no alteration in gene expression or coding sequence between normal B6 and *Sle1a.1* T cells, but rather found a novel isoform, Pbx1-d, expressed only in *Sle1a.1* T cells. Sequence analysis revealed that Pbx1-d lacks the DNA binding domain associated with Pbx1-a and Pbx1-b. Microarray analysis revealed an alteration in the retinoic acid signaling pathway in *Sle1a.1* $CD4^+$ T cells. Treatment of B6.*Sle1a.1* CD4-expressing cells that do not express CD25 with IL-2, TGF-β, and retinoic acid resulted in a decreased adaptive Treg (aTreg) population when compared to normal B6 CD4-expressing cells lacking CD25 expression. These results indicate that Pbx1 controls the level of Tregs in the periphery in a retinoic acid-dependent fashion.

CHAPTER 1
INTRODUCTION

Systemic lupus erythematosus is a chronic autoimmune disease with varied pathogenicity from mild forms to fatal end organ damage. It manifests itself in auto-antibody production directed primarily against nuclear antigens (ANAs). The most serious clinical outcome results from immune complex deposition in the kidney, which eventually leads to autoimmune lupus glomerulonephritis (GN), and finally complete kidney failure. Genetic predisposition has been implicated in susceptibility to SLE in the past 20 years. For inherited diseases, the variation in disease expression resulting from genetic factors reflects variation in genome structure, called a polymorphism (1). The difference in genome structure can be assumed to be the cause, either directly or indirectly, for the change in function observed. Alterations in genome structure can occur in many ways, such as an alteration in protein structure encoded by a gene or an alteration in the level of expression of a gene (1). An individual polymorphism can then be inherited from parent to offspring according to Mendelian laws. By identifying functional variations associated with SLE, we can make advancements in understanding the root causes of disease development and progression by pinpointing fundamental disease mechanisms.

Spontaneous murine models of SLE offer insights into genetic susceptibility, as disease development has been unequivocally dependent on genetic predisposition. Our lab focuses on congenic dissection of the NZM2410 spontaneous murine model of SLE. The NZM2410 strain is a recombinant inbred line derived from the NZB and NZW strains (2-4). In short, by converting a polygenic system, the NZM2410 strain in our case, into a group of monogenic strains, each carrying a susceptibility locus on a resistant genetic background, one can analyze, both genetically and functionally, these monogenic strains for traits to associate with a particular genetic defect. The NZM2410 susceptibility loci are referred to as *Sle1*, *Sle2*, and *Sle3*

corresponding to chromosomes 1, 4 and 7, and combine to confer the same phenotype as the NZM2410 (3). Major lupus susceptibility locus *Sle1*, associated with multiple murine models of SLE as well as human patients (5-11), comprises three independent loci, *Sle1a*, *Sle1b*, and *Sle1c* (12), and has been shown to enhance activation levels and effector functions of $CD4^+$ T cells, reduce the size of the $CD4^+ CD25^+ CD62L^+$ Treg subset, as well as decrease expression of the transcription factor *Foxp3* among this population preceding autoantibody production (13).

It was initially proposed to study one of three susceptibility loci contained within the *Sle1* susceptibility locus, *Sle1a*, by characterizing its contribution to SLE disease as well as further refining the genetic map in an effort to narrow down the list of genes within the *Sle1a* locus potentially involved in SLE susceptibility and pathogenesis. A review of the literature will be presented, followed by the first chapter of results. In this first chapter, we will examine the entire *Sle1a* region's effect and show that its expression affects a genetic pathway regulating production of Tregs and responses to Tregs in a manner that is not restricted to tolerance to nuclear antigens. We will accomplish this by characterizing the phenotypes of the T cells, effector and regulatory, as well as the antigen-presenting cells (APCs), in both *in vitro* and *in vivo* settings. The identification of the *Sle1a* gene(s) will therefore uncover a novel and broad pathway by which autoreactive T cells are regulated by Tregs.

In the second results chapter, we will further examine *Sle1a* to refine the genetic map in order to locate genes responsible for previously observed phenotypes. *Sle1a* contains at least two susceptibility loci, and by examining the effects of expression of these truncated regions of *Sle1a*, we can observe phenotypes that will either co-segregate with the parental strain (*Sle1a*) or require the synergistic involvement of the other locus. We will accomplish this by performing the same experiments from the first results chapter in mice containing truncated regions of *Sle1a*,

called *Sle1a.1* and *Sle1a.2*, and show that none of the phenotypes observed for the entire *Sle1a* region can be fully explained by expression of either of the individual subloci alone, indicating that the synergistic effect of the two subloci is a requirement for *Sle1a*-mediated action.

Finally, in the third results chapter, we will further examine one of *Sle1a*'s subloci, *Sle1a.1*, to elucidate its involvement in SLE pathogenesis. We will show that this locus contains only one gene, *Pbx1*, which encodes for pre-B cell leukemia transcription factor, and that the NZW allele of this gene not only leads to alternate isoforms of the protein, but leads to alterations in expression of a number of other genes using microarray data analysis. We will carry out functional studies to elucidate a potential mechanism of Pbx1's action and hypothesize that expression of the NZW allele of *Pbx1* leads to decreased production of adaptive regulatory T cells (aTregs), thereby contributing to the observed phenotype of decreased Tregs attributable to expression of the entire *Sle1a* locus.

CHAPTER 2
LITERATURE REVIEW

Systemic Lupus Erythematosus in Humans

An estimated 1.5-2 million Americans currently suffer from some form of SLE according to the Lupus Foundation of America, with this disease most notably affecting women between the ages of 15 and 45. SLE onset involves a combination of factors including genetic predisposition relating to the occurrence of susceptibility loci as well as environmental triggers. This disease has a concordance rate of 2-5% for dizygotic twins compared to 24-58% for monozygotic twins, leading to a 10-fold difference (14). Siblings of SLE-affected individuals have also been shown to be 20 times more likely to develop SLE than those without affected siblings (15). There is also wide disease variation among different ethnic populations regarding disease prevalence with a 3-4 times greater prevalence in African-American or Afro-Caribbean populations compared with Caucasians in the same location (16,17).

Linkage analysis and association analysis have been the two techniques employed to study the genetics of human lupus (18). Linkage studies look for the segregation of linkage of particular genetic markers with disease in the family members of SLE-affected individuals. These markers are generally linked to disease by causing variants due to their relative proximity on the genome and the capability of being inherited together at meiosis rather than being functional themselves (1). If many families present with this same marker, it can be inferred that there is a disease causing variant located within that particular area of the genome, called a linkage region (1). Association studies involve the selection of a candidate gene marker, with a variation already known and implicated in disease susceptibility (1). The frequencies of these variations or polymorphisms are assessed between SLE-affected individuals and appropriate controls (19). If a polymorphism has a greater than expected frequency in SLE-affected

individuals as compared to controls, it can be inferred that this variant is disease-causing. Until recently it was a problem that only a small number of markers could be studied because a hypothesized gene or set of genes was required to begin study (1). Fortunately, in the past six years, advances in technology have made it possible to type thousands of single nucleotide polymorphisms (SNPs) across the whole genome at the same time (20,21). Recent Genome Wide Association scans have implicated several genes as contributors to SLE susceptibility (22-25) . *TNFAIP3* encodes for TNFAIP3, a key regulator of NF-κB signaling through ubiquitin modification of adaptor proteins RIP and TRAF6 downstream of TNFα and Toll-like receptors, respectively (26,27). *BANK1* is specifically expressed in B cells and has a potential role in B cell receptor-mediated activation (28,29). *IRF5* encodes for IFN regulatory factor 5 and is a transcription factor downstream of the type I IFN and Toll-like receptors (30-33). *ITGAM* encodes for CD11b, combines with the β_2 chain to form a leukocyte integrin, CR3, that is important for adherence to neutrophils and monocytes to stimulated endothelium, and is also a receptor for the complement component C3 degradation product, iC3b (34).

While many linkage regions have been identified by performing multiple whole genome scans in several cohorts of lupus families, an issue arises because these regions are not found in all studies. It is therefore necessary to follow the approach of analyzing the results using a rigorous significance cut-off (35) or pooling results from multiple studies in a meta-analysis (36,37) to eliminate false positives and find priority regions on which to focus (1). However, linkage has proven to be rather poor in identifying candidate genes due to in SLE due to inconsistencies between studies caused by a number of issues (1). The first is that there are many types of lupus. The American College of Rheumatology requires only 4 of 11 criteria to be diagnosed with SLE (38), and this may cause inconsistencies in what genes lead to particular

symptoms or phenotypes. The second issue is genetic heterogeneity. It may be that different SLE susceptibility genes act in different ethnic groups. It has been shown in mice that susceptibility genes act in different ways depending on the genetic background as a result of modifying genes elsewhere in the genome (39). The third, and most interesting in our case, is that detection of genes that only modestly affect the phenotype of interest is difficult using linkage analysis (40). Of interest for our study is the idea of the "common variants/common disease" hypothesis by Becker, which implicates multiple genetic loci interacting with one another in an additive manner leading to overall disease susceptibility, while individually having only small effects (41). These issues lead to the necessity of other methods to elucidate genes involved in susceptibility to SLE.

Murine Models of Systemic Lupus Erythematosus

A number of inbred mouse models which develop SLE-like disorders have been used to further our understanding of SLE genetics. However, caution must be taken when attempting to locate human candidate genes from mouse studies. Although 99% of murine genes are considered to have a homologous human gene, it cannot be assumed that the gene functions in the same manner and must be investigated, if possible, in a human setting to definitively prove its importance (42). There are three general types of mouse models used at present to define genetic susceptibility to SLE: gene manipulation-derived, induced, and spontaneous. While these models share the common features of ANAs and autoantibody- and/or immune-complex mediated end-organ disease, they differ in severity, autoantibody profile, sex predominance and clinical manifestations (43). Gene-manipulation-derived models include the FcγRIIb-deficient mice (44), while induced-disease models include the procainamide, pristane, idiotype, mercury-induced autoimmunity (DBA/2, resistance) (45) and the *Mycobacterium bovis*-induced systemic autoimmunity in NOD mice (46,47), mapping studies have only been performed in the latter two

(47,48). Induced models have provided insights as to the relationship between genetic susceptibility and environmental factors (43). The NZB, NZW, MRL-*Fas*^{lpr}, and the BXSB strains are the most commonly studied among the spontaneous models. Extensive studies have also been carried out on recombinant inbred lines derived from the NZB and NZW strains, the NZM2328 strain and, in the case of our lab, the NZM2410 strain (2-4).

In order to study these mice to locate potential candidate genes, one can follow either the reverse or forward genetics approaches. Reverse genetics involves genetic manipulation via transgenes or site-directed mutagenesis (gene knockout) of known genes in nonautoimmune strains, thereby revealing specific gene alterations which are capable of promoting SLE development (43). One can also employ this strategy to identify modifier genes that can reduce susceptibility by genetically manipulating SLE-prone strains by backcrossing of mainly knockout genes (43). Forward genetics involves identification of genes based solely on chromosomal region by initially mapping traits to chromosomal regions or loci, generating and characterizing locus-containing interval congenic mice, subsequently narrowing the interval by screening congenic mice with smaller-sized intervals, and then finally the screening of candidate genes (43). This is the method employed by our lab. This approach may lead to difficulties in identifying susceptibility genes should strong linkage disequilibrium exist in the region of interest and in verifying the role of even the most appealing of candidate genes (43). These difficulties can only be worse in the case of human SLE, highlighting mouse models as an important complementary tool for confirming the significance of gene variation.

Identification of Susceptibility Loci

Since publication of a landmark paper detailing the linkage analysis of type I diabetes in the NOD mouse (49), the desire to locate susceptibility genes for murine models of autoimmune diseases has grown. Our lab has focused its attention on the identification of SLE-susceptibility

loci in the NZM2410 spontaneous murine model using genome-wide linkage analysis (or "Quantitative Trait Locus" mapping) in an effort to discover the genes involved in susceptibility. The NZM2410 susceptibility loci are referred to as *Sle1*, *Sle2*, and *Sle3* corresponding to chromosomes 1, 4 and 7 (3), and combine in the B6.*Sle1Sle2Sle3* triple congenic strain to confer the same phenotype as the NZM2410 (50,51). By studying the single, double and triple congenic strains containing either one, two or all three susceptibility loci from the NZM2410 bred onto a normal B6 background, distinct phenotypes as well as necessary interactions between multiple loci for full disease development have been observed. In both humans and mice, one of the hallmarks of SLE is the loss of tolerance to nuclear antigens, most notably chromatin and anti-dsDNA (10), leading to the production of nuclear antigen specific autoantibodies or ANAs. This particular phenotype has been linked to the *Sle1* locus.

The *Sle1* locus has been identified as having the strongest statistical linkage to clinical disease and nephritis (3), it maps to a genomic region (telomeric chromosome 1 and 1q23-1q42) that has been linked to other mouse models (52) as well as human SLE patients (53), and has been implicated as the root of a multi-step process leading to SLE pathogenesis and disease onset based on the following results (53). This locus leads to a selective loss of tolerance to chromatin, with the production of serum IgG antibodies preferentially targeting H2A/H2B/DNA subnucleosomes as early as 5 months of age, with a penetrance of more than 75% (54,55). Expression of *Sle1* has been shown to be necessary for nephritis development in the NZM2410 model (4,51). In addition, a highly penetrant clinical pathology was observed when *Sle1* was coexpressed with various single mutations or other *Sle*-susceptibility loci (44,50,51,56). Mixed bone marrow chimeras have shown that *Sle1* expression leads to increased expression of activation markers, cytokine production and generation of histone-specific T cells, with the

activation of T cells occurring independent of B cell presence indicating an intrinsic T cell defect (54,57). Based on results from *Sle1* recombinants, the production of ANAs maps to at least three independent loci, referred to as *Sle1a*, *Sle1b*, and *Sle1c* (12).

The genetic regions *Sle1b* and *Sle1c* currently possess candidate genes, while no candidate gene exists at present for the *Sle1a* region (58,59). It was proposed that *Sle1b* is allelic with a cluster of seven genes from the SLAM/CD2 family, with the strongest candidate being *Ly108* (59). It has now been shown that the normal isoform of Ly108 has the capacity to sensitize immature B cells to deletion and *RAG* re-expression, indicating that Ly108 is a potential regulator of tolerance checkpoints, censoring self-reactive B cells and thereby safeguarding against autoimmunity (60). However, the NZW allele of *Ly108* leads to an alternative isoform lacking this regulatory capacity (60). Cr2 encodes for the complement receptors type 1 and 2 (CR1/CR2) and is a strong candidate gene for *Sle1c* due to a mutation disrupting its function as a B cell receptor (58). The following studies concentrate on the *Sle1a* sub-congenic region of *Sle1* by examining a strain carrying a truncated region of the centromeric end of *Sle1a*, termed B6.*Sle1a.1*, and a strain which includes the telomeric end of *Sle1a*, termed B6.*Sle1a.2* to further characterize this important locus in an attempt to elucidate its role in the production of autoreactive T cells corresponding to a reduction in Tregs. Expression of *Sle1a* in T cells has been shown to lead to levels of activation as well as cytokine production similar to that of the entire *Sle1* locus (13). Adoptively transferred T cells from both *Sle1* and *Sle1a* were shown to be capable of providing help to chromatin-specific B cells (13). It was inferred that expression of *Sle1a* alone was necessary and sufficient to induce autoreactive T cells capable of providing help to chromatin-specific B cells to produce ANAs (13). It was also observed that expression of

Sle1a was associated with a reduced level of $CD4^+$ $CD25^+$ Tregs preceding autoantibody production, potentially suggesting a causal relationship with autoreactive T cell generation (13).

Tregs and Systemic Lupus Erythematosus

T cells play an important role in the development of SLE, both in mice and in humans (61). Normally, central deletion by negative selection of T cells reactive to self-peptide occurs in the thymus. This prevents interactions of these autoreactive T cells and self-antigen in the periphery, thereby averting an autoimmune state. Should these cells escape deletion, mechanisms exist in the periphery to keep these interactions in check, including extrinsic regulation by Tregs and intrinsic regulation inherently programmed into the cells themselves based on life spans, etc. However, in certain cases, these checks are not fully functional and autoimmunity results. In the case of murine models, the disease state cannot be initiated without the help of T cells, and these cells can also be directly pathogenic, forming part of the cellular infiltrates in skin and interstitial areas of the kidney. Although it is evident that T cells are necessary for disease, the degree to which intrinsic defects in these cells contribute to disease remains unclear (62).

According to Sakaguchi, nearly 30 years ago studies done by Nishizuka & Sakakura (63) and Penhale et al. (64) started the current interest in T cell-mediated control of self-reactive T cells as being a key dominant mechanism involved in self-tolerance (65). And so began the interest in Tregs, which were initially defined as $CD4^+$ $CD25^+$ T cells. Substantial evidence exists showing the indispensable role of Tregs in the maintenance of natural self-tolerance and negative control of immune responses capable of leading to pathogenesis (65). Landmark experiments done by Sakaguchi revealed that removal of $CD4^+$ $CD25^+$ T cells elicited autoimmune disease as well as enhancing immune responses to non-self antigens through the development of autoimmune $CD4^+$ T cells resulting in the breakdown of B cell self-tolerance as

well (66). Tregs are detectable in the periphery of normal mice at day 3 post-birth, and rapidly increase to the adult level (5-10%) around 3 weeks of age (67).

The mode by which Tregs perform their suppressive function involves recognition of and stimulation by self-antigen to control self-reactive T cells in the normal internal environment either by cell-cell contact or by cytokine-mediated pathways, and are capable of suppressing not only T cells with the same antigen specificity, but also those specific for other antigens once activated (68).

Foxp3 encodes for Scurfin, a member of the forkhead/winged-helix family of transcription factors, which has been implicated in the onset of autoimmune disease when defective (69). Loss-of-function mutations of this transcription factor leads to impaired Treg development, the lethal X-linked lymphoproliferative disorder of the naturally arising scurfy mouse strain (70-73), and the homologous disorder in humans, IPEX, or 'immune dysregulation, polyendocrinopathy, enteropathy, X-linked' syndrome (74-76). This molecule has also been implicated in the development and function of Tregs due to studies which revealed that transduction of *Foxp3* induced expression of CD25, CTLA-4, CD103, and GITR, which are closely related to the function of Tregs, as well as suppressing proliferation *in vitro* of other T cells and preventing autoimmune development *in vivo*, thus giving rise to the idea that *Foxp3* is the master control gene for the development and function of Tregs (77). Studies have shown that Treg effector function but not necessarily lineage commitment requires the expression of functional Foxp3 protein (78), and that continued expression is needed to maintain the transcriptional and functional program established during Treg cell development (79). Interestingly, it was found that Foxp3 expression leads to distinct transcriptional programs for those cells produced in the thymus compared to those developing in the periphery (80). High expression of CD62L on

Tregs has also been implicated as important due to tissue-homing capabilities (81). Based on these and other studies, a specific panel of markers will be used to analyze phenotypic differences of Tregs among the chosen mice strains.

Tregs are not only produced in the thymus, called natural Tregs (nTregs), but several types of *in vivo*- and *in vitro*-induced Tregs have been described, called inducible Tregs (iTregs). The difference between these populations and nTregs is that the iTregs are dependent on cytokines. What is unknown is whether these populations arise from a common precursor. There are the Tr1 cells, which produce high levels of immunosuppressive IL-10, can be generated by chronic activation in the presence of IL-10 in both mice and humans, and are able to prevent autoimmune colitis in an experimental model (82,83). The Th3 cells were observed after oral or intravenous antigen application and produce TGF-β (84). Similar to the Tr1 cells, these cells can mediate their suppressive capacity through IL-10 secretion as well in a cell-contact independent fashion (84). Induction of alloantigen-specific Tregs can be accomplished by immature DCs expressing low levels of costimulatory molecules *in vitro* (85). The Tregs produced under these conditions are similar to Tr1 cells in that they produce high levels of IL-10, but act like nTregs that are cell-contact dependent. The human $CD4^+ CD25^+$ natural-like Tregs act in a cytokine-independent manner, but can drive $CD4^+ CD25^-$ T cells to develop a cytokine-dependent immunosuppressive capacity (86). These natural-like Tregs are formed by rendering $CD4^+$ T cells from peripheral blood anergic with alloantigeneic stimulation in the presence of TGF-β. $CD4^+ Foxp3^-$ naïve T cells have been shown to be capable of conversion to a $CD4^+ Foxp3^+$ Treg population, called adaptive Tregs (aTregs), which present with the same phenotypic and suppressive characteristics as nTregs both *in vivo* and *in vitro* (87-91). It has also been shown that this conversion not only requires IL-2, but is dependent on TGF-β (87,92,93). These aTregs have been used a means of

inducing tolerance in a number of settings, including inflammatory bowel disease and allotransplantation models (88-90,94). Clinical use can perhaps be achieved by improving the potency of these aTreg treatments.

While the loss of Tregs can result in organ-specific autoimmune diseases (81,95), little is known about Tregs in the systemic autoimmune disease, SLE. The role of Tregs in prevention of systemic autoimmunity has been shown in double transgenic models expressing a TCR and its cognate antigen (96-98). The generation of antigen-specific Tregs was found to be necessary in the (SWRxNZB)F1 model for tolerance induction to nuclear antigen with histone peptide (99). Spontaneous models for lupus are less understood, however. The NZM2328 lupus model showed no spontaneous global deficiency upon analysis of neonatal thymectomy experiments (100), mixed results have been shown for other models (100,101). One study showed that adoptive transfer of Tregs can ameliorate disease phenotypes induced by SLE (102), although these Tregs were exogenously expanded. Our lab has shown that introduction unmanipulated $CD4^+$ $CD25^+$ Tregs isolated from the B6.*Sle1Sle2Sle3* mice into B6.*Sle1Sle2Sle3* recipients before the onset of autoantibody production can reduce all disease phenotypes, indicating that the number of Treg plays an important role in systemic autoimmunity.

Studies initially done in human SLE reported decreased percentages of $CD4^+$ $CD25^+$ T cells in the peripheral blood of SLE patients as compared to controls (103). However, results from these studies were inconclusive due to discrepancies in accurately discriminating the Treg population from activated T cells since Tregs in humans are restricted to the $CD25^{high}$ fraction. nTregs were shown to be lower in number and functionally impaired during active SLE in several studies (104-107), with $Foxp3^+$ Tregs shown to be present in reduced numbers in pediatric patients with SLE (108). Of interest for our study is the result that $CD4^+$ $CD25^{high}$ T

cells isolated from SLE patients shared the same phenotypic and functional characteristics with normal Tregs from healthy controls (106), suggesting that the number of Tregs and not their function was an important aspect of disease progression. This same study proposed that the SLE Tregs were present at lower numbers due to increased sensitivity to Fas-mediated apoptosis. Another study also showed that there were reduced numbers of suppressive $CD4^{+}$ $CD25^{high}$ Tregs in the peripheral blood of patients with active SLE, and that these cells presented with decreased levels of Foxp3 as well as decreased suppressive function *in vitro* (109). However, Tregs in the peripheral blood of patients with inactive SLE were essentially normal. This same study showed that Tregs from the patients with active SLE were also capable of being restored to the normal phenotype *in vitro*, indicating that the possibility to reverse the deficit of Tregs was a possibility, as shown by another group (110).

Retinoic Acid and the Immune System

Retinoic acid (RA), the most active metabolite of vitamin A, and its derivatives regulate a variety of cellular functions including development, proliferation, differentiation, immune function and death in multiple cell types via two specific families of nuclear receptors functioning as ligand-inducible transcription factors, the retinoic acid receptors (RAR) and the retinoic X receptors (RXR) (111-114).

In the immune system, RA plays important roles in the functional regulation of many immune cell types (115). RA has been shown to prevent activation-induced cell death of T cells and inhibits Th1 while enhancing Th2 responses (116,117). RA enhances cytotoxicity and T cell proliferation, most likely mediated by enhancing IL-2 secretion and signaling in T cells (118,119). In vivo, RA suppresses inflammatory responses and tissue damage in addition to ameliorating symptoms in a variety of autoimmune diseases in animal models, including experimental autoimmune encephalomyelitis, rheumatoid arthritis, type I diabetes, inflammatory

bowel disease, and SLE (120-124). RA treatment has been shown to ameliorate symptoms in both the NZB/WF_1 and MRL-*Fas*^{lpr} murine models of SLE through intraperitoneal injection or dietary supplement, respectively (121,125,126), although the mechanism by which it mediates this protection has not been fully elucidated. This RA-mediated protection has been thought to be due to inhibition of Th1 responses. RA produced by $CD103^+$ dendritic cells (DCs) has recently been implicated in inducing aTregs in the gut (127-130) while inhibiting Th17 differentiation (129). Apparently, RA has a dual role in the maintenance of tolerance in that it favors induction of Treg cells and has the capacity to either block or enhance Th17 differentiation depending on its concentration (131). How this action is mediated has not been addressed but is of great interest in elucidating the mechanism by which RA suppresses autoimmunity.

The Noelle group showed that a unique population of aTregs can be generated with RA treatment *in vitro* that is capable of homing to the small intestine lamina propria *in vivo* (127). RA was shown to potently synergize with TGF-β in driving Foxp3 induction, even in the presence of high levels of co-stimulation, thereby increasing the frequency of these aTregs as well their durability (127). RA's effect has been shown to be independent of IL-2, signal transducer and activator of transcription 5 (Stat5) and Stat 3 (132). RA has been implicated in enhancing TGF-β signaling by increasing the expression and phosphorylation of Smad3, which then binds with Smad4, and this complex can then translocate to the nucleus, thereby resulting in increased Foxp3 expression (133) and therefore and increase in Tregs.

Pbx1 and the Immune System

Pre-B cell leukemia transcription factor 1 (Pbx1) is a member of the TALE (three-amino-acid-loop extension) family of homeodomain proteins. Pbx1 was first implicated in pre-B cell leukemia in 1990 as a chromosome 1:19 translocation (134,135) which resulted in an E2A-Pbx1

fusion protein (homeodomain of Pbx1 and the transcriptional activation domain of E2A) that alters normal transcriptional regulation by Pbx1 (114). Pbx1 contains the following domains: the meis binding domain (MIM), the nuclear localization signal (NLS), the hox binding domain (HCM), the PBC homeodomain (PBC-A,B), and the homeo DNA binding domain (HD).

Pbx1 can interacts with multiple Hox proteins and this complex can enhance both Hox DNA-binding specificity and affinity (136-139). Both Meis and Prep, additional TALE proteins, can bind the Pbx1-Hox dimer, resulting in a trimeric complex capable of further transcriptional regulation due to its higher DNA binding specificity (140-143). Both Meis and Prep associate with Pbx1 in the cytoplasm and induce a conformational change in Pbx1, exposing the nuclear localization signal, and subsequently causing translocation of the dimeric protein complex to the nucleus (144). Pbx1 and Meis have also been implicated as essential cofactors for optimal binding of MyoD to DNA, thereby demonstrating another activity for these cofactors independent from Hox-related transcription (145). Treatment with RA has been shown to expand both Meis and Pbx1 expression in various cell types (114,146,147).

Pbx1 has been implicated in B cell development and has been described as a necessary factor in very early B cell commitment (148). It was demonstrated in mice that Pbx1 plays a key role in pancreatic development, and that it is an important cofactor for the master regulator of pancreatic development and function, Pdx1 (149). Based on a study done by the Ma group and published in 2007, Pbx1, and more specifically Pbx1-b, was shown to be a physiologically critical mediator of apoptotic cell-induced IL-10 gene transcription and IL-10 cytokine production by macrophages, with its transcriptional role found to be uncoupled from phagocytosis (150).

There is 100% amino acid sequence homology between mouse and human Pbx1. It has been shown to be a strong biological candidate gene for type 2 diabetes at the chromosome 1q21-q24 susceptibility locus in humans (151). A small case-control study was done in which 20 *Pbx1* variants were evaluated for association with type 2 diabetes in Utah Caucasians, with only three variants shown to be nominally associated, as were haplotypes involving intron 2 variants (152).

CHAPTER 3
MURINE LUPUS SUSCEPTIBILITY LOCUS *SLE1A* CONTROLS REGULATORY T CELL NUMBER AND FUNCTION THROUGH MULTIPE MECHANISMS

Introduction

[1]Dominant suppression through regulatory T cells, and specifically $CD4^+$ $CD25^+$ T cells (Tregs) expressing the Foxp3 transcription factor, has now been accepted as a major mechanism by which self-tolerance is maintained. A decrease in Treg numbers or function has been directly associated with autoimmune pathogenesis in multiple diseases and their associated mouse models (153,154). Reduced numbers of Tregs (106,108) as well as defective Treg function (109) have more recently been described in lupus patients. Tregs play a key role in maintaining tolerance to DNA in a transgenic mouse model (96). In spontaneous models of lupus, tolerance induction was dependent on the generation of $Foxp3^+$ Tregs in (NZB X SWR)F_1 (99) and (NZB X NZW)F_1 (BWF1) mice (155), and Treg transfers showed a significantly protective effect in BWF1 mice (102). Other murine studies have documented a Treg protective effect for autoantibody production, but not for end-organ pathology (101,156). While these studies have documented a role for Tregs in controlling at least some aspects of lupus pathogenesis, they did not determine the mechanisms responsible for the observed Treg deficiency in either number or function.

We have used NZM2410-derived congenic strains to address these questions. The major lupus susceptibility locus *Sle1* controls tolerance to nuclear antigens (10,55) and intrinsically affects both B and T cells (57,157). Multiple loci contribute to the *Sle1* phenotype (12), and we have shown *Sle1a* and *Sle1c* are largely responsible for the generation of autoreactive T cells,

[1] *Reprinted with permission from The Association of American Immunologists, Inc. Copyright 2007.* Cuda, C. M., S. Wan, E. S. Sobel, B. P. Croker, and L. Morel. 2007. Murine lupus susceptibility locus *Sle1a* controls regulatory T cell number and function through multiple mechanisms. *J Immunol* 179:7439.

with *Sle1a* alone accounting for $CD4^+$ T cell phenotypes equivalent to that of the entire *Sle1* locus (13). $CD4^+$ T cells expressing *Sle1a* show significantly increased levels of activation and proliferation, as well as increased production of cytokines. Furthermore, purified *Sle1a* $CD4^+$ T cells are able to induce *in vivo* the production of anti-nuclear antibodies (Abs) from either *Sle1* or normal B cells (13). Finally, *Sle1a* is associated with a reduction of $CD4^+$ $CD25^+$ $CD62L^+$ $Foxp3^+$ Treg numbers (13). Conversely, the B6.*Sle1.Sle2.Sle3* (B6.TC) strain, which reconstitutes the full autoimmune pathogenesis with the three major NZM2410 susceptibility loci (51), produces dendritic cells (DCs) that prevent Treg inhibitory functions on effector T cells (Teffs) (158). Production of high amounts of IL-6 by B6.TC DCs is a major mechanism by which this interference occurred, and we have shown that this phenotype maps to *Sle1* (158).

In this study, we examined the functional consequences of *Sle1a* expression on Tregs and cells directly interacting with them. Treg function can be affected by multiple factors, including their number and intrinsic function. Many studies have reported a critical role of accessory cells, especially DCs, for optimal Treg development and function (159), and imaging studies have clearly shown that Tregs exert their regulatory function through direct contact with DCs (160,161). Teffs can also be resistant to suppression, as was shown in the MRL/lpr model of lupus (162). The complexity of a regulatory system in which these three cellular compartments play a critical role requires a model in which each the compartment can be assayed independently in a syngeneic / autologous fashion (163). The NZM2410 congenic strains, which share over 96% of their C57BL/6 (B6) genome, offer such a model. By comparing the B6.*Sle1a* congenics to B6 controls, we first confirmed that *Sle1a* results in a reduced subset of $CD4^+$ $CD25^+$ $CD62L^+$ $Foxp3^+$ cells. *Sle1a* Tregs, however, appeared normal regarding expression of markers commonly associated with the regulatory phenotype, and were capable of normal

regulatory activity at high Treg:Teff ratios. *Sle1a* also induced an increased level of activation in $CD4^+$ T cells and DCs, and both of these compartments significantly interfered with Treg regulatory function. Finally, we showed that the activated $CD4^+$ T cell phenotypes and reduced Treg numbers required *Sle1a* expression in these T cells, suggesting that the generation of autoreactive T cells results from additive intrinsic defects in both *Sle1a* expressing $CD4^+$ T cells and DCs. Overall, these results identify *Sle1a* as a locus playing a major role in T cell tolerance through Treg regulation by multiple mechanisms.

Materials and Methods

Mice

C57BL/6J (B6), C57BL/6J-Cg-IghaThy1aGpila/J (B6.*Thy1a*), and B6.129S7-*Rag1*^{tm1Mom}/J (B6.$Rag^{-/-}$) mice were originally obtained from The Jackson Laboratory. The B6.NZM2410-*Sle1* (B6.*Sle1*) congenic strain contains a 37-cM NZM2410-derived interval defined by the D1MIT101 and D1MIT155 markers (164). The B6.NZM2410-*Sle1a* (B6.*Sle1a*) sub-congenic line represents a 2.96 Mb interval between and excluding D1MIT370 and D1MIT147 (12,13). Unless specified, experiments were conducted with 8-12 month old female congenic mice and B6 matched controls. This age is past the induction of anti-nuclear Abs and autoreactive cells in most B6.*Sle1* and B6.*Sle1a* mice (12,55). All mice were bred and maintained at the University of Florida in specific pathogen-free conditions. All experiments were conducted according to protocols approved at the University of Florida Institutional Animal Care and Use Committee.

Flow Cytometry

Briefly, cells were first blocked on ice with staining buffer (PBS, 5% horse serum, and 0.09% sodium azide) supplemented with 10% rabbit serum and pretreated with anti-CD16/CD32 (2.4G2) to block FcR-mediated binding. Cells were then stained with pre-titrated amounts of the following FITC-, PE-, allophycocyanin-, or biotin-conjugated Abs: CD4 (RM4–5), CD69

(H1.2F3), CD25 (7D4), CD62L (MEL-14), GITR (DTA-1), CD103, ICOS (CD278 clone 7E.17G9), B220 (RA3-6B2), CD3 (145-2C11), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD40 (HM40-3), CD62L, CD80 (16-10A1), CD86 (GL1), I-A^b (AF6-120.1), NK1.1 (PK126), TER119, mPDCA-1 (Miltenyi Biotec) or isotype controls. All antibodies were from BD Biosciences unless otherwise specified. A combination of PE-conjugated anti-CD3, CD19, NK1.1, and TER119 antibodies were used to exclude $CD11c^{low}$ T cells, B cells, natural killer cells and erythroblasts, respectively. Biotin-conjugated Abs were revealed using streptavidin-PerCP Cy5.5 (BD Biosciences). Intracellular expression of CD152 (CTLA-4) and IL-10 was analyzed in fixed permeabilized cells with Cytofix/Cytoperm Plus kit (BD Pharmingen). For IL-10 expression, splenocytes were cultured in the presence of anti-CD3 and anti-CD28 (1 μg/ml) for 72 hours, and intracellular IL-10 levels in $CD4^+$ $ICOS^+$ cells were assessed by flow cytometry. IL-10 was also measured in the culture supernatant using an OptEIA Mouse IL-10 ELISA kit (BD Pharmingen) according to manufacturer's instructions. Foxp3 expression was determined using an intracellular Foxp3-PE staining kit (eBioscience). Cell staining was analyzed using a FACSCalibur (BD Biosciences). At least 50,000 events were acquired per sample, and dead cells were excluded based on scatter characteristics. Positive staining was determined as equal to or greater than the top 5% of the isotype control.

Suppression Assays

$CD4^-$ (antigen presenting cell [APC]), $CD4^+$ $CD25^-$ Teff and $CD4^+$ $CD25^+$ Treg populations were purified from splenocytes with magnetic beads using the $CD4^+$ $CD25^+$ Treg cell kit according to the manufacturer's instructions (Miltenyi Biotech), and cultured in 96-well flat-bottomed plates in the presence of 1 μg/ml of anti-CD3 to assess in vitro suppression levels of Tregs. Teffs and Tregs FACS analysis consistently showed >90% purity. The number of Teffs was kept constant at 5×10^5 cells per well, whereas the number of Tregs was titrated using

four-fold dilutions. Cultures were maintained for 54 hours before pulsing with 1 μCi/well of [^{3}H]-thymidine for an additional 18 hours. Cells were then collected onto fiber filtermats with a PHD cell harvester (Cambridge Technology) and counted using a Beta-scintillation counter. To assess the suppressive function of Tregs *in vivo*, $CD4^+$ $CD25^-$ Teff and $CD4^+$ $CD25^+$ Treg populations from 2 month old female donor mice were purified with magnetic beads and transferred into age-matched female B6.$Rag^{-/-}$ mice by injection into the tail vein. Recipients received 4 x 10^5 B6 or B6.*Sle1a* Teffs in the presence or absence of B6 or B6.*Sle1a 1* x 10^5 Tregs. After injection, mice were monitored for clinical signs of colitis for up to 8 weeks, and body weight was monitored weekly. Mice that lost 15% or more of body weight, or showing overt clinical signs of disease were sacrificed. Routine colon, stomach and kidney histology was performed to compare B6 and B6.*Sle1a* Teffs and Tregs functions and scored blindly in a semi-quantitative fashion. Colon multiplicative score (0 - 81) was calculated by multiplying the thickness score by the infiltrate score in both the mucosa and the muscularis. The kidney additive score (0-4) was computed by adding 1 to the infiltrate score for the presence of granulomas.

Generation of DCs and DC Phenotyping

DCs were generated from bone marrow (BM) with GM-CSF and IL-4 (R&D Systems) as previously described (158). To assess activation levels and cytokine production, BM-derived $CD11c^+$ DCs were cultured for 24 hours with LPS (Sigma) at 1 μg/ml. The supernatants were harvested and stored at – 80 ^oC until assayed with commercial ELISA kits (BD Pharmingen).

Bone Marrow Chimeras

Chimeras were prepared as previously described (157). In brief, 6-8 week old female B6 mice were lethally irradiated with two doses of 525 Rad γ irradiation (4 hours apart) in a Gammacell 40 ^{137}Cs apparatus (MDS Nordion). Donor BM cells were depleted of mature T cells

using CD5 Microbeads (Miltenyi Biotech). Production of mixed BM chimeras was performed at a 1:1 ratio for the B6.*Thy1a* and B6.*Sle1a* strains. Ten million cells were given to each mouse by tail vein injection. Chimeric mice were maintained for 8 weeks, and lymphocytes were analyzed by flow cytometry to evaluate proliferation, activation, and Treg levels. The B6.*Thy1a* and B6.*Sle1a* origin of the T cells was determined with CD90.1 (*Thy1a*) and CD90.2 (*Thy1b*). $CD4^+$ cellular proliferation was measured by staining splenocytes with 2.5 μM CFSE (Molecular Probes) prior to stimulation with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) and culture for 48 hours in a 37°C, 5% CO_2 incubator. Activation was measured by staining lymphocytes with CD4, and CD69 after 12 hours of anti-CD3 and anti-CD28 stimulation. Treg levels were measured by staining lymphocytes with CD4, CD25, and CD62L prior to culture.

Statistical Analysis

Unpaired t test statistics (one- or two-tailed as indicated) were used to compare the phenotypes of the B6.*Sle1* and B6.*Sle1a* strains with that of B6. Comparisons for BM chimeras were made with paired two-tailed Student's t tests after verification that the data was normally distributed with GraphPad Prism 4. Nonparametric Mann-Whitney tests were used when the data were not normally distributed. Comparisons for colon and kidney pathology were made with one-way ANOVA tests. Each in vitro experiment was performed at least twice with reproducible results.

Results

Sle1 and *Sle1a* are Associated with Increased Levels of Activated T Cells and Decreased Levels of Tregs

Previous results indicated that *Sle1* is associated with a significantly increased number of activated $CD4^+$ T cells (57,165) as well as a decreased number of $CD4^+$ $CD25^+$ Tregs, and that this phenotype mapped to *Sle1a* and to a lesser extent to *Sle1c* (13,166). We confirmed these

results by showing that congenic mice expressing *Sle1* or *Sle1a* showed a significant increase in activated $CD4^+$ $CD69^+$ or $CD4^+$ $CD44^+$ T cells, and a significant decrease in naïve $CD4^+$ $CD62L^+$ $CD44^-$ T cells as compared to B6 (data not shown). In addition, we show here that both *Sle1* and *Sle1a* $CD4^+$ T cells showed a significantly increased expression of ICOS (Fig. 3-1A), which is a co-stimulatory molecule that is pivotal for T-B interactions and highly expressed on follicular helper T cells (167). We further analyzed $CD4^+$ $ICOS^+$ cells by culturing total splenocytes in the presence of anti-CD3 and anti-CD28 to assess intracellular levels and secreted IL-10. We observed a trend of increased levels of $CD4^+$ $ICOS^+$ $IL\text{-}10^+$ cells as well as production of IL-10 in the culture supernatant associated with *Sle1a*, but not to a statistically significant degree (data not shown). B6.*Sle1a* congenic mice also showed significantly decreased percentages of $CD4^+$ $CD25^+$ $CD62L^{hi}$ (Fig. 3-1B), with significantly less $CD4^+$ $CD25^+$ cells expressing CD62L, indicating that this locus induced a higher proportion of recently activated cells $CD4^+$ $CD25^+$ cells as opposed to Tregs. These findings were confirmed by intracellular expression of Foxp3 (Fig. 3-1C and D). It is of note that $CD4^+$ $CD25^+$ $CD62L^{hi}$ cells have lost the $Foxp3^{hi}$ peak in the B6.*Sle1* and B6.*Sle1a* mice, suggesting that this population contains less functional $Foxp3^+$ Tregs in these mice than in B6. Similar results were obtained for younger mice ranging from 5 to 7 months of age (data not shown). Overall, these results confirm that *Sle1a* expression increases the number of activated T cells and diminishes the $Foxp3^+$ Treg compartment. However, the expression of markers commonly associated with Tregs, namely GITR, CD103, and CTLA-4, was not affected by *Sle1a* expression in both $CD4^+$ $CD25^+$ (Fig. 3-2A) and $CD4^+$ $CD25^+$ $CD62L^+$ populations (Fig. 3-2B), suggesting that *Sle1a* Tregs may be functional, although reduced in numbers.

Sle1a Tregs Require a Higher Treg:Teff Ratio to Support Inhibition to a Level Equivalent to B6 Tregs

We assessed *Sle1a* Treg function using standard suppression assays in which the proliferation of $CD4^+$ $CD25^-$ Teffs was measured in response to anti-CD3 stimulation in the presence of APCs and graded ratios of Tregs. In these assays, the only variable was the Treg origin, B6 or B6.*Sle1a*, while all other cells were of B6 origin. As shown in Fig. 3-3, there was no difference between the inhibitory capability of *Sle1a* and B6 Tregs at a 1:1 Treg:Teff ratio. A significantly diminished inhibitory function appeared however at 1:4 and 1:16. At this latter ratio, inhibition by *Sle1a* Tregs was no longer observed, and in some cases increased proliferation was observed with *Sle1a* Treg addition, as we have previously reported for B6.TC Tregs (158). This titration result is consistent with the $CD4^+$ $CD25^+$ population containing a smaller proportion of functional Tregs in B6.*Sle1a* mice.

Sle1a Expression Increases DC Activation

We have recently shown that B6.TC DCs display an abnormal phenotype and hinder Treg function in an IL-6 dependent manner (158). Furthermore, elevated IL-6 production coupled with Treg inhibition mapped to *Sle1*. We show here that the *Sle1a* mediates an expansion of $CD11c^+$ $CD11b^+$ $B220^-$ myeloid mDCs in the spleen (Fig. 3-4A) and lymph nodes (data not shown). Plasmacytoid pDCs gated as $CD11c^+$ $B220^+$ (Fig. 3-4A), but not as $B220^+$ $PDCA\text{-}1^+$ (data not shown), were also modestly expanded in B6.*Sle1a* spleens. In addition, *Sle1a* DCs displayed a significantly increased expression of activation markers as shown for CD86 (Fig. 3-4C) and CD80 (Fig. 3-4D) that is similar to that of *Sle1* DCs. These *ex-vivo* phenotypes were age-dependent as they reached statistical significance only in old mice. Increased levels of activation markers such as CD40 and CD86 (Fig. 3-4B), or CD80 and class II MHC (data not shown), and increased production of IL-6 (Fig. 3-4E) and IL-12 (Fig. 3-4F) were readily

obtained by LPS stimulation of DCs derived from either young (2-3 months old) or old B6.*Sle1a* BM. These levels were similar to what we have previously described for B6.*Sle1*. Overall, these results show that *Sle1a* induces an age-dependent DC accumulation in secondary lymphoid organs, and that these DCs produce more inflammatory cytokines than that of the B6 controls. We have previously reported that *Sle1* increases activation of peripheral B cells (157,168). Here we show that *Sle1a* splenic B cells also expressed a significantly higher level of CD19, CD80 and CD86 in old mice (data not shown). Overall, these results show that *Sle1a* increases activation not only in $CD4^+$ T cells but also in DCs and B cells.

Sle1a Expression Affects the Ability of Both DCs to Support Treg Suppression and Teffs to be Inhibited

Given that *Sle1a* expression affects all cellular compartments in a suppression assay, namely Tregs, Teffs and APCs, we investigated the consequences of *Sle1a* expression independently in each of these cellular compartments on the ability of Tregs to suppress Teff proliferation (Fig. 3-5). As seen earlier, expression of *Sle1a* in Tregs had a significant effect on Treg function at a low Treg:Teff ratio. Interestingly, *Sle1a* expression in Teffs significantly hindered the action of Tregs, although this effect was no longer significant at the 1:16 Treg:Teff ratio. Expression of *Sle1a* in APCs significantly prevented inhibition at all three ratios, and even induced enhanced proliferation at the 1:16 Treg:Teff ratio. This latter effect was observed with DCs from B6.TC mice (158), suggesting that the *Sle1a* locus plays a major role in the DC defective functions in this model. In conclusion, *Sle1a* expression in either one of the 3 members of the suppression assay significantly impacts the ability of Tregs to suppress Teff proliferation.

We also assessed *in vivo* the effect of *Sle1a* expression on effector and regulatory $CD4^+$ T cell function in a rapid model of disease adapted from the experimental colitis model (169). B6.$Rag^{-/-}$ mice received 4 x 10^5 $CD4^+$ $CD25^-$ Teffs from either 2 month old B6 or B6.*Sle1a* in the

presence or absence of 1 x 10^5 $CD4^+$ $CD25^+$ Tregs from B6 or B6.*Sle1a* mice. As expected, B6 Teffs induced weight loss and colitis, which was abrogated by the presence of B6 Tregs (Fig. 3-6 and 3-7A and B). *Sle1a*-expressing Tregs did not suppress B6 Teff function as well as B6 Tregs, possibly due to a lower ratio of functional Tregs within the injected $CD4^+$ $CD25^+$ population. We also observed that the *Sle1a*-expressing Teffs are resistant to suppression by either B6 or *Sle1a*-Tregs. In addition to lymphocytic infiltrates, *Sle1a* Teffs resulted in the presence of elevated numbers of polymorphonuclear neutrophils (PMN) in the lesions, leading to cryptitis (Fig. 3-6C2). Finally the combination of *Sle1a* Teffs and Tregs led to the most severe pathology with the presence of giant cells (Fig. 3-6D2). Interestingly, similar results were observed in the kidneys (Fig. 3-7C and data not shown), but not in the stomach. Teffs induced interstitial inflammation and granulomas in B6.Rag kidneys, which were suppressed by B6 Tregs only when the Teffs were of B6 origin. As in the colon, *Sle1a* Teffs were also associated with giant cells in the kidneys, indicating a greater level of inflammation. Overall, this *in vivo* experiment confirmed that the *Sle1a* $CD4^+$ $CD25^+$ population is less effective at suppressing Teff functions and that *Sle1a* Teffs are resistant to Treg suppression.

Sle1a Expression Intrinsically Affects $CD4^+$ T Cell Phenotypes

The results presented above showed that *Sle1a* expression affects the function of multiple hematopoeitic cell compartments, which prompted us to examine whether *Sle1a* expression was required for $CD4^+$ T cells to show the functional defects reported above. To address this question, we produced mixed bone marrow chimeras by injecting T cell-depleted bone marrow cells from either normal B6.*Thy1a* or B6.*Sle1a* (*Thy1b*) donor mice into lethally irradiated B6 hosts. As shown in Fig. 3-6, the increased proliferation and activation of $CD4^+$ T cells, as well as the decreased percentage of Tregs were completely reproduced by *Sle1a* bone-marrow-derived cells (compare B6.*Thy1a*→B6 and B6.*Sle1a*→B6). More interestingly, in mixed

chimeras containing both *Sle1a*-expressing and normal $CD4^+$ T cells, only those T cells expressing *Sle1a* displayed enhanced proliferation, as measured by *in vitro* CFSE dilution (Fig. 3-8A), and activation, as measured by CD69 expression (Fig. 3-8B). Corresponding histograms show expression of levels of both CFSE on gated $CD4^+$ T cells (Fig. 3-8A and B, respectively). Conversely, the percentage of $CD62L^+$ Treg was significantly lower in *Sle1a*-expressing T cells than in B6 and can be visualized in the corresponding histogram depicting CD62L levels on $CD4^+$ $CD25^+$ gated cells (Fig. 3-8C). Taken together, these results show unambiguously that *Sle1a* results in intrinsically activated $CD4^+$ T cells. *Sle1a* expression in non-hematopoeitic cells is not required for induction of these phenotypes. The abnormal phenotypes are not transferable to bystander normal T cells, excluding *Sle1a* being mediated through a soluble factor.

Discussion

We have shown here that expression of *Sle1a* is sufficient to induce increased activation levels of $CD4^+$ T cells, DCs and B cells, as well as to down-regulate Treg levels. We also show that *Sle1a* $CD4^+$ T cells express increased levels of the co-stimulation marker ICOS, which has been shown to play a critical role in T cell help to B cells, especially in germinal centers (170,171). Elevated ICOS expression on T cells from lupus patients has now been reported in three independent studies (172-174). These last two studies reported that ICOS stimulation of lupus T cells significantly enhanced anti-dsDNA Ab production from autologous B cells, which is equivalent to what we have shown for *Sle1a* T cells, which were able to induce anti-chromatin production in both autologous *Sle1a*-expressing B cells and normal B cells (13). These results also suggest that *Sle1a* confers a T cell phenotype that is found in lupus patients, which further validates the need to identify the identity of the *Sle1a* gene(s). Future experiments should address the specific role of ICOS in this process. High levels of ICOS have been associated with IL-10 secretion by $CD4^+$ T cells (175), and IL-10 production by $CD4^+$ T cells is significantly

increased in the NZM2410 model (176). There was however no consistent increase of IL-10 production by *Sle1a* $CD4^+$ T cells, suggesting that another mechanism may be involved.

Sle1a induces a reduction in the size of the Treg compartment, but these cells express normal levels of CTLA-4, CD103, and GITR, molecules which have been commonly associated with the regulatory phenotype. In addition, at the higher ratios of Treg:Teff, *Sle1a*-expressing Tregs are fully capable of suppressing the proliferation of B6 Teffs on a per-cell basis in the presence of B6 APCs. However, at lower ratios of Treg:Teff, this suppressive capability is decreased, consistent with a reduced proportion of functional Tregs within the $CD4^+CD25^+$ T cell population of the B6.*Sle1a* mice. In addition to *in vitro* suppression assays, we also performed adoptive transfers adapted from the experimental model of colitis to test the *in vivo* effect of *Sle1a* on Treg and Teff functions in a rapid model of disease. Results from the *in vivo* study confirmed our *in vitro* data. We cannot exclude, however that *Sle1a* also affects Treg inhibitory functions. Indeed, a recent construct with a non-functional Foxp3 has demonstrated that the expression of Treg signature makers can develop normally in cells that completely lack inhibitory functions (177). A definitive answer to that question will require breeding of *Sle1a* to a Foxp3 reporter construct, which we are currently pursuing.

While we have shown that the *Sle1a*-expressing Tregs are capable of suppression, in situations where either the Teffs or the APCs express *Sle1a*, the suppressive capability of normal B6 Tregs is significantly reduced, suggesting that the *Sle1a* locus confers a resistance to suppression of Teff proliferation and that the APCs are playing a role in this phenomenon. It is of note that the APC population used in our *in vitro* suppression assays contains not only DCs but B cells as well. We have previously shown the effects of *Sle1a* DCs on Treg suppression (19), however, *Sle1a* affects both of these cell types. This indicates a potential role of activated

B cells on Treg function, and is an avenue to be studied further. A similar Teff resistance has been previously reported in another model of lupus (162), but it is not clear at this point whether this resistance is the mere consequence of hyperactivation, or a result of involvement with a specific mechanism. *Cbl-b* deficiency results in a resistance to Treg regulation involving TGF-β, and this mutation also induces an increased level of activation in effector T cells (178). To our knowledge, no other mechanisms of resistance to Tregs have been reported and additional experiments will be necessary to determine how *Sle1a* confers this resistance in $CD4^+$ T cells. We have previously shown that DCs from the NZM2410 triple congenic strain B6.TC prevent Tregs from performing their inhibitory functions, primarily through the production of IL-6 (158). We report here that *Sle1a*-expressing DCs present the same phenotype of high IL-6 production and Treg inhibition, indicating that this locus plays a major role in the overall DC phenotype of lupus-prone mice. Interestingly, the type-1 diabetes prone NOD mice, which have a reduced number of Tregs (179,180), also produce APCs that fail to fully support Treg functions (180). These results suggest that defective support or active inhibition of Treg functions by DCs may be a common mechanism affecting autoimmune pathogenesis.

Mixed BM chimeras have shown here that the increased proliferation and activation of *Sle1a*-expressing T cells, as well as the reduced *Sle1a* Treg level require that *Sle1a* be expressed in these T cells. These results differ from what might have been predicted from the *in vitro* reconstitution experiments shown in Figure 3-5, where B6.*Sle1a*-derived APCs inhibited Treg function. The BM chimera results do not mean that *Sle1a* exclusively affects $CD4^+$ T cells. In an analogous set of experiments, BM chimeras showed that T cell activation and autoreactivity mediated by *Sle3* were the indirect result of *Sle3* expression within the myeloid compartment (62,181). It is therefore possible that the *Sle1a*-induced intrinsic defects in $CD4^+$ T cells are

indirectly responsible for the DC and B cell abnormalities. Alternatively, the *Sle1a* gene(s) may control a pathway present in all three cellular compartments. In any event, indirect or direct activation of DCs by *Sle1a* was not sufficient to convey extrinsic changes to B6-derived $CD4^+$ T cells *in vivo*. The exact cause for these differences is unclear, and highlights the need to confirm *in vitro* findings with *in vivo* studies. Additional mixed BM chimeras will be necessary to address whether *Sle1a* expression in these DCs and B cells is necessary for production of the activated phenotypes.

Autoreactive T cells are a feature common to many autoimmune diseases for which a genetic basis has been demonstrated, yet only very few genes have been identified as responsible for this phenotype (182). In addition to *Cbl-b* discussed above (178), null alleles of *Gadd45a* (183) or *E2f2* (184) result in a lower threshold for T cell activation culminating in autoimmune pathogenesis, while null alleles in *Ctla4* (185) and *Foxp3* (186) result in massive inflammatory and autoimmune responses through the disruption of the Treg compartment. More recently, a natural polymorphism in the *Il2* gene has been identified as responsible for the diabetes susceptibility locus *Idd3* in the NOD mouse, also through an impairment of Treg function (187). The *Sle1a* interval does not contain any gene with obvious function in T cells. Our *in vitro* results showed that *Sle1a* confers an autoimmune phenotype to $CD4^+$ T cells in the colon, which is not typically associated with lupus pathogenesis. This indicates that *Sle1a* affects a genetic pathway regulating production of Tregs and responses to Tregs in a manner that is not restricted to tolerance to nuclear antigens. The identification of the *Sle1a* gene(s) will therefore uncover a novel and broad pathway by which autoreactive T cells are regulated by Tregs.

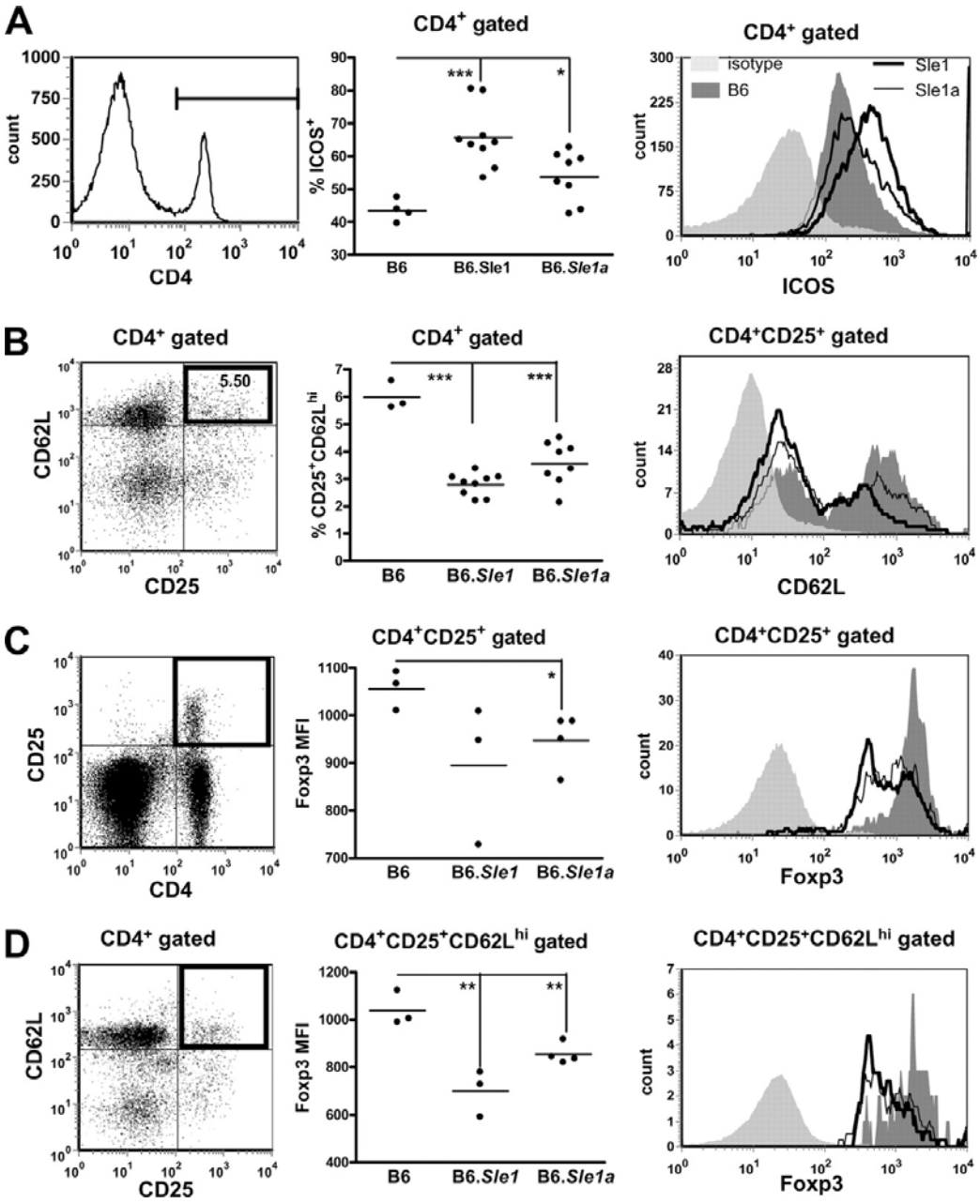

Figure 3-1. *Sle1a* results in increased $CD4^+$ T cell activation and a diminished Treg compartment. Splenocytes from 8-12 month old B6, B6.*Sle1*, and B6.*Sle1a* mice were labeled for surface CD4, ICOS, CD25, and CD62L and intracellular Foxp3 expression and analyzed by fluorescence-activated cell sorting. Each point represents an individual animal. Representative gating on a B6 mouse is shown in the left-hand column (marker for A, and rectangular gate for B-D), and representative expression levels in all three strains are shown in the right-hand column. The light grey filled histograms show isotype controls, dark grey filled histograms show B6 values, while thick and thin black lines represent B6.*Sle1* and B6.*Sle1a*, respectively. All comparisons were performed with B6 values. Two-tailed *t* tests: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

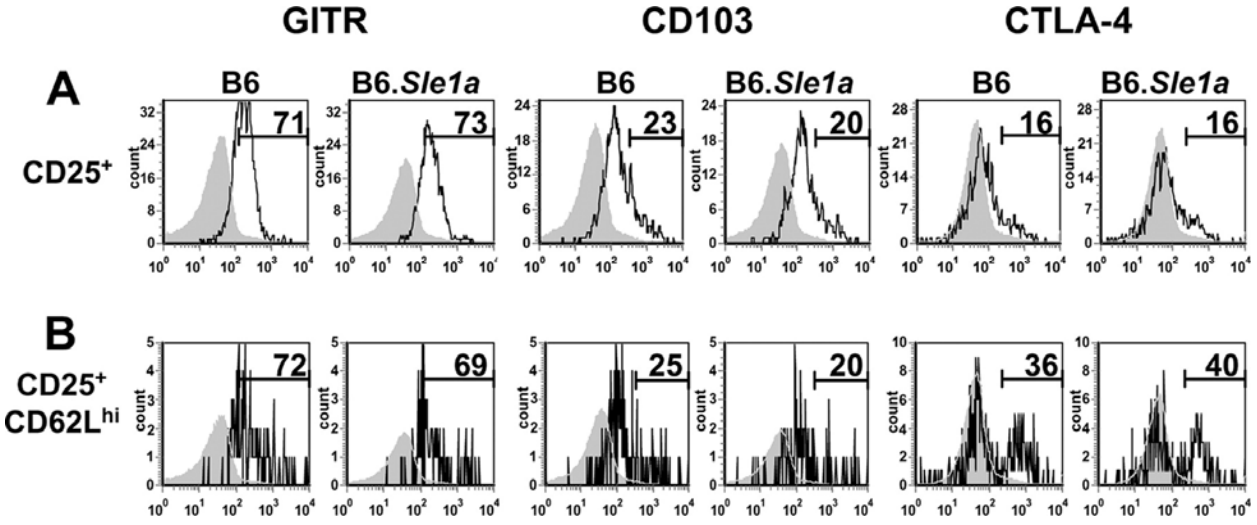

Figure 3-2. *Sle1a* does not affect expression of markers associated with the regulatory phenotype. $CD4^+ CD25^+$ (A) or $CD4^+ CD25^+ CD62L^+$ (B) splenocytes from B6 and B6.*Sle1a* mice were compared for GITR, CD103 or intracellular CTLA-4 expression. Representative histograms of at least 5 mice per strain are shown. The percentage of positive cells (based on the isotype control shown by the grey histograms) is indicated above each marker.

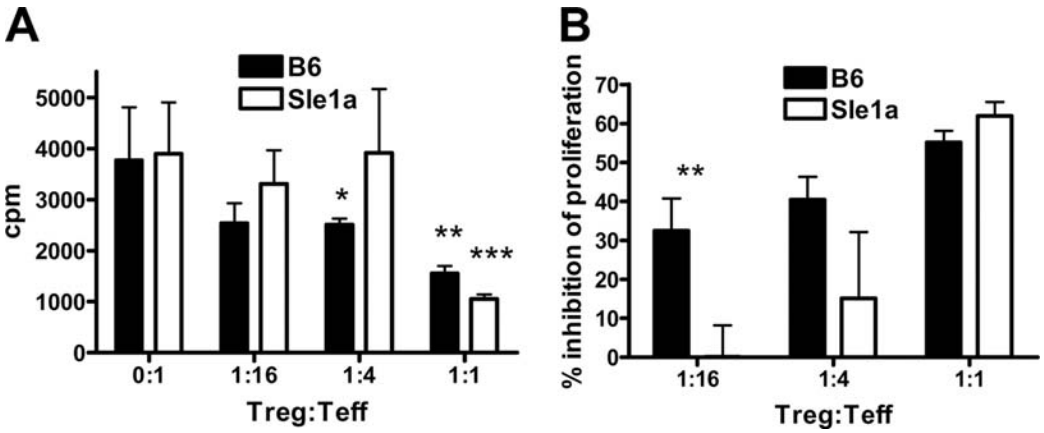

Figure 3-3. *Sle1a* Tregs are deficient in inhibiting proliferation at low Treg:Teff ratios. Inhibition of proliferation assays were set up with B6-derived APCs and Teffs, and either B6 (black bars) or B6.*Sle1a* (white bars) Tregs at the indicated ratio. A) Representative assay comparing proliferation in the presence of B6 or *Sle1a* Tregs (6 mice per strain). Means and standard errors, and results of one-tailed *t* tests between the 0:1 assays and the various Treg:Teff ratios for each strain. B) Normalized percentage inhibition of proliferation of the 0:1 assays at the various Treg:Teff ratios for each strain combined from three different assays (15 mice per strain). Means and standard errors, and results of one-tailed *t* tests between the two strains for each Treg:Teff ratio. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

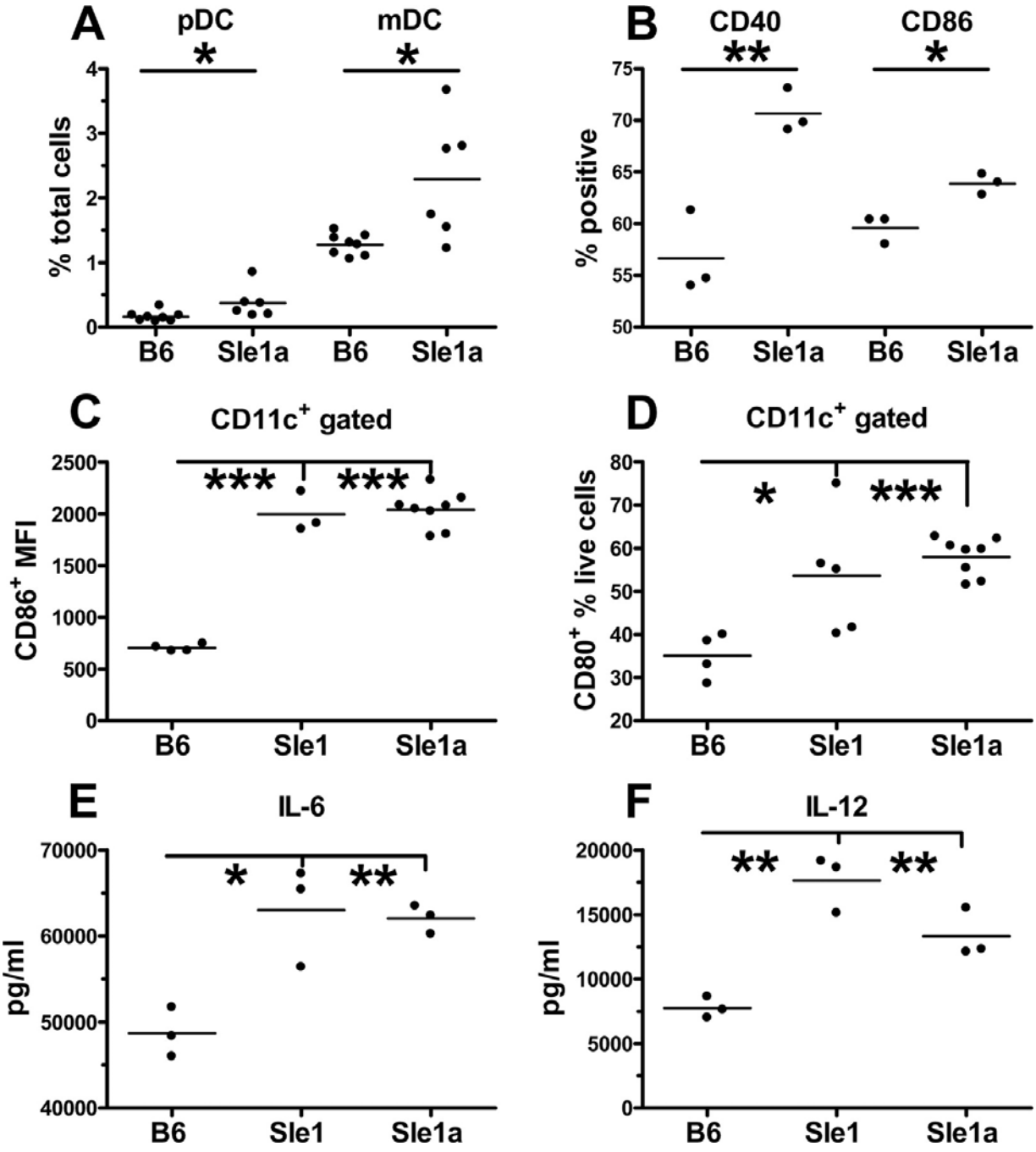

Figure 3-4. *Sle1a* expression activates dendritic cells. A) pDCs and mDCs were significantly expanded in the spleen of B6.*Sle1a* mice as compared to B6. B) CD40 and CD86 were significantly up-regulated in B6.*Sle1a* BM-derived DCs in response to LPS. Splenic DCs were significantly more activated in B6.*Sle1* and B6.*Sle1a* than in B6 mice, as shown by CD86 (C) and CD80 (D) expression. DCs derived from B6.*Sle1* or B6.*Sle1a* BM produced significantly more IL-6 (E) and IL-12 (F) than B6 after LPS exposure. Each point represents an individual animal. All comparisons were performed with B6 values. Two-tailed *t* tests: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

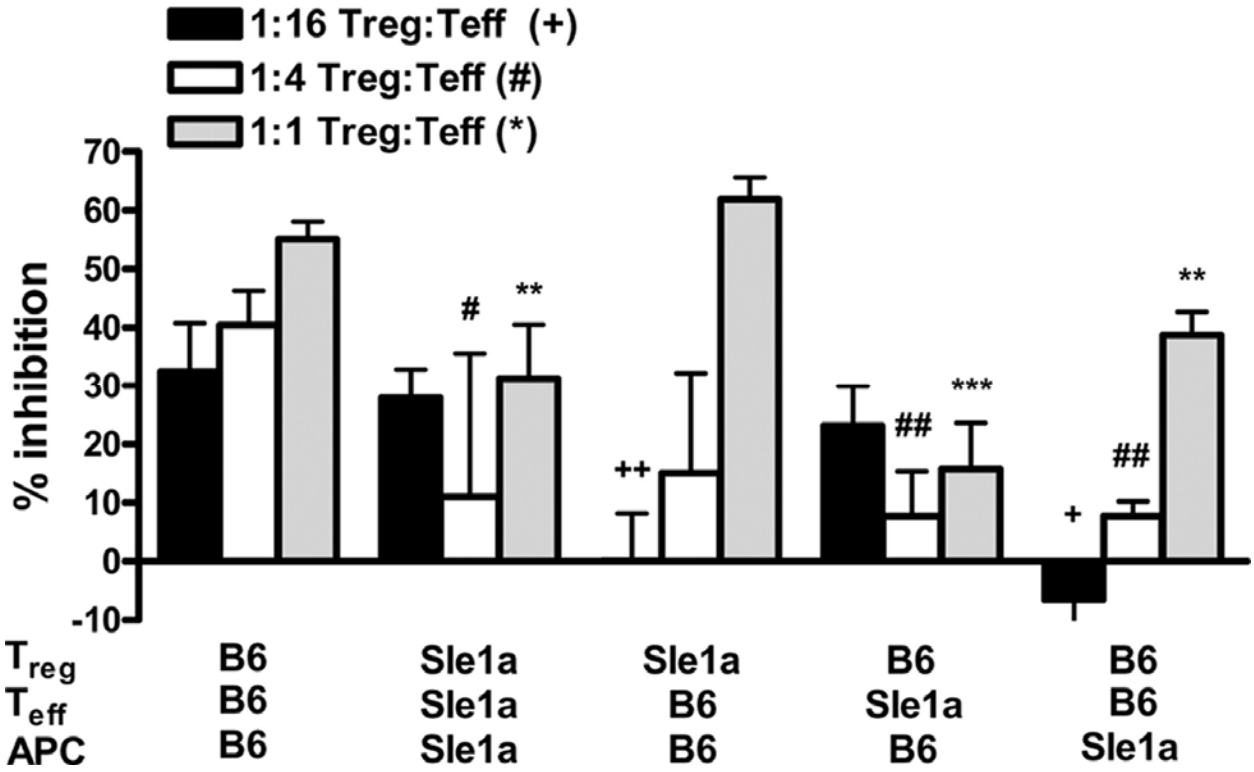

Figure 3-5. *Sle1a* expression in Tregs, Teffs, or APCs affects the extent of the inhibition of Teff proliferation. The inhibition of $CD4^+$ $CD25^+$ Teff proliferation in presence of 1:1, 1:4, or 1:16 Treg:Teff ratios is expressed as a percentage of the proliferation induced in the absence of Tregs for each condition. The origin, B6 or B6.*Sle1a*, of Tregs, Teffs, and APCs is indicated under each column. The graphs show the means and standard errors of three independent assays with 3-4 mice per strain at 6 months of age in each assay. Results of one-tailed *t* tests between each condition and the "all B6 condition" are indicated for each Treg:Teff ratio. $^{+,\#,}$ *: $p<0.05$, $^{++,\#\#,}$ **: $p<0.01$, $^{+++,\#\#\#,}$ ***: $p<0.001$.

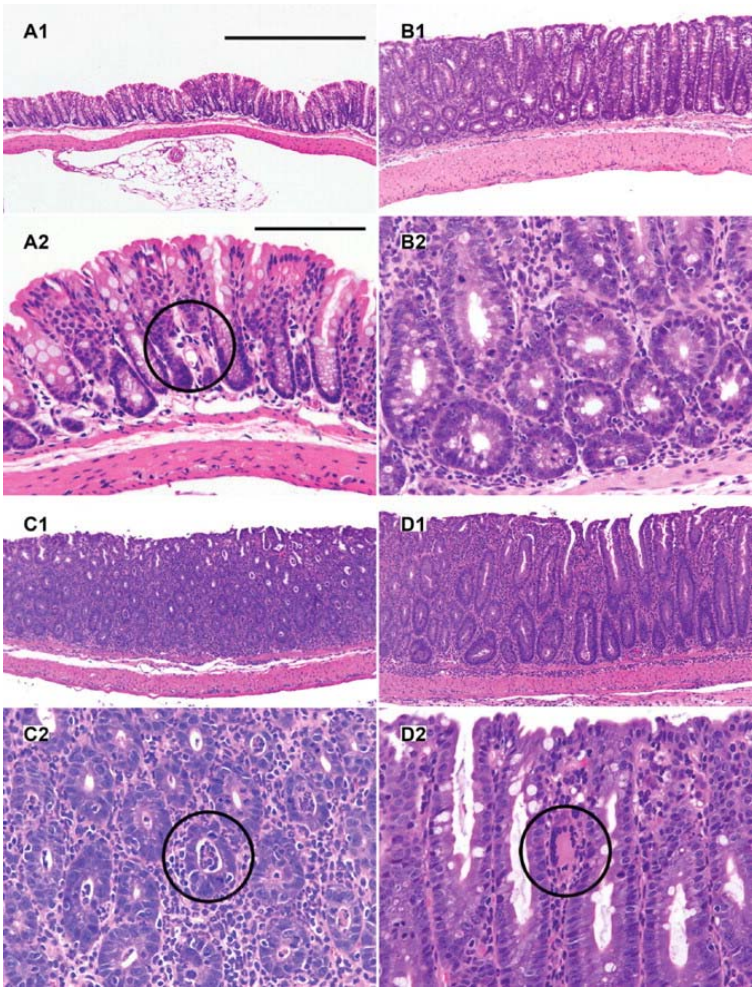

Figure 3-6. *Sle1a* expression in either Tregs or Teffs affects the extent of disease in an adoptive transfer model. $CD4^+$ $CD25^-$ Teff and $CD4^+$ $CD25^+$ Treg populations from 2 mo old female donor mice were transferred into age-matched female B6.$Rag1^{-/-}$ recipient mice. Representative colon histology: All parts of the composite labeled 1 are the same magnification (0.5 mm) and all parts labeled 2 (0.1 mm) are 4 times those labeled 1. Each two sub-figures with the same letter (e.g. A1 & 2) are from the same animal. A1 & 2) B6 Teffs and B6 Tregs. The figure is representative of the control group with normal thickness and minimal lymphocytic infiltrate in the lamina propria (A2, circle, center). B1 & 2) B6 Teffs and *Sle1a* Tregs. The figure is representative of about 2-fold increase in thickness (B1). There is a notable increase in lamina propria thickness and mononuclear cell infiltrate. There are also increases in epithelial infiltrating lymphocytes, epithelial apoptosis and mitosis (B2). C1 & 2) *Sle1a* Teffs and B6 Tregs. This figure is representative of an additional increase in thickness (C1). In addition to the lymphocytes and other findings noted above, increased PMNs are present in the lamina propria and glands (cryptitis and crypt abscess, C2, center). D1 & 2) *Sle1a* Teffs and *Sle1a* Tregs. This is the greatest overall thickness with inflammation extending into the muscularis propria (D1, bottom). There is an increase in PMN and lymphocytes through out the mucosa. Occasional multinucleated giant cells are present (D2, circle).

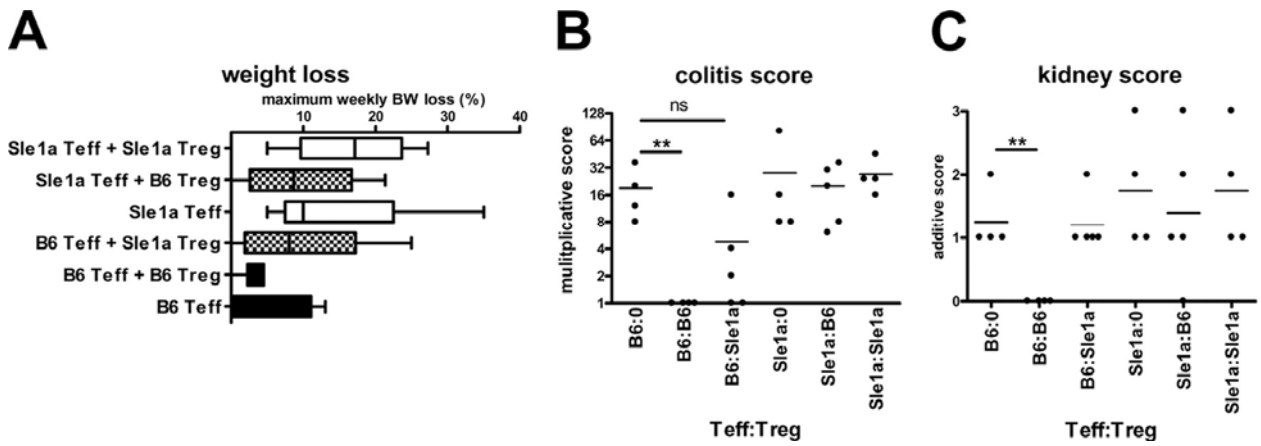

Figure 3-7. Quantification of the effects of $CD4^+$ $CD25^-$ Teff and $CD4^+$ $CD25^+$ Treg transfers into B6.Rag1$^{-/-}$ mice. A) Maximum weekly percentage of body weight loss up to 8 weeks after transfer. The box and whisker plot shows the medians, 25 and 75 percentiles and minima and maxima for each group. B) Multiplicative colitis pathology score. C) Additive kidney pathology score (infiltrate score + giant cell presence). In B and C, the strain of origin of Teff and Tregs is indicated on the X axes, with 0 indicating the absence of Tregs. ANOVA: *: $p<0.05$.

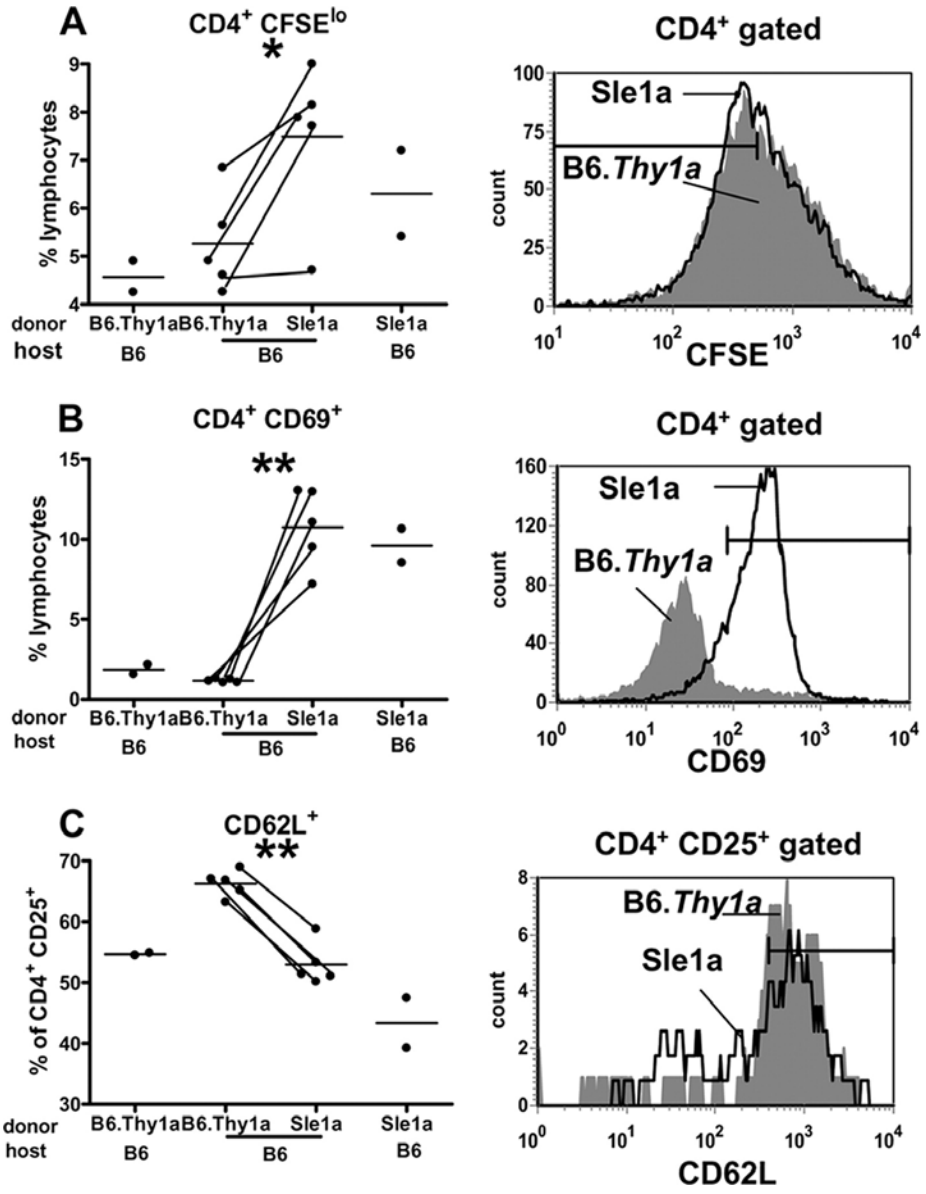

Figure 3-8. *Sle1a* expression affects $CD4^+$ function in a cell-intrinsic manner. B6 hosts were reconstituted with B6.*Thy1a* and / or B6.*Sle1a* BM. Connected samples indicate values for $CD4^+$ T cells expressing the *Thy1a* (CD90.1-gated) or *Thy1b* in B6.*Sle1a* mice (CD90.2-gated) alleles within the same mouse. Controls are represented by B6.*Thy1a*→B6 and B6.*Sle1a*→B6 single-strain BM transfers. A) *In vitro* anti-CD3 induced proliferation measured as the percentage of $CD4^+$ $CFSE^{low}$ lymphocytes with representative histogram showing CFSE expression on gated $CD4^+$ T cells. B) Activation measured as the percentage of $CD4^+$ $CD69^+$ lymphocytes with representative histogram showing CD69 expression on gated CD4+ T cells. C) Treg levels, measured as the percentage of $CD4^+$ $CD25^+$ splenocytes expressing CD62L with representative histogram showing CD62L expression. Each point represents an individual animal. Two-tailed *t* tests: *: $p<0.05$, **: $p<0.01$. Data representative of two independent set of BM chimeras with 5 mixed chimeras in each.

CHAPTER 4
THE CONTROL OF REGULATORY T CELL NUMBER AND FUNCTION BY MURINE LUPUS SUSCEPTIBILITY LOCUS *SLE1A* REQUIRES THE SYNERGISTIC EFFECT OF SUBLOCI *SLE1A.1* AND *SLE1A.2*

Introduction

In the previous chapter, we presented results which showed that expression of *Sle1a* results in increased levels of activation of T and B cells as well as DCs. This overall increased activation was accompanied by a decreased Treg compartment. These phenotypes were shown to be due to a T cell-intrinsic defect. On a per-cell basis, the *Sle1a*-expressing Tregs were capable of normal suppressive function, although there were less of them, but the *Sle1a*-expressing Teffs were resistant to suppression and the *Sle1a*-expressing APCs play a role in this mechanism. Now that we have phenotypes attributable to *Sle1a* expression, it is of interest to dissect out which phenotypes co-segregate with the truncated regions of *Sle1a*, *Sle1a.1* and *Sle1a.2*. Once we can determine the regions to which specific *Sle1a* phenotypes map, we can narrow down the genes leading to SLE susceptibility.

Materials and Methods

Mice

The production of B6.*Sle1a*$^{NZW/NZW}$ (B6.*Sle1a*) was described as B6.*Sle1a(15-353)* (12) and B6.*Sle1a.2*$^{NZW/NZW}$ (B6.*Sle1a.2*) mice as B6.*Sle1(111-148)* (188). B6.*Sle1a.1*$^{NZW/NZW}$ (B6.*Sle1a.1*) was obtained as a recombinant interval from B6.*Sle1a* (Fig. 4-1). C57BL/6J (B6), C57BL/6J-Cg-IghaThy1aGpila/J (B6.*Thy1a*), and B6.129S7-*Rag1*^{tm1Mom}/J (B6.Rag$^{-/-}$) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME). Unless specified, experiments were conducted with 8-12month old female congenic mice and B6 matched controls. This age is past the induction of anti-nuclear Abs and autoreactive cells in most B6.*Sle1* and B6.*Sle1* sub-congenic mice (12,55). All mice were bred and maintained at the

University of Florida in specific pathogen-free conditions. All experiments were conducted according to protocols approved by the University of Florida Institutional Animal Care and Use Committee.

Sle1a Map

PCR genotyping with microsatellite markers was performed as previously described (12) and SNP genotyping was performed by direct sequencing.

Flow Cytometry

Briefly, cells were first blocked on ice with staining buffer (PBS, 5% horse serum, and 0.09% sodium azide) supplemented with 10% rabbit serum and pretreated with anti-CD16/CD32 (2.4G2). Cells were then stained with pre-titrated amounts of the following FITC-, PE-, allophycocyanin-, or biotin-conjugated Abs: CD4 (RM4–5), CD69 (H1.2F3), CD25 (7D4), CD62L (MEL-14), ICOS (CD278 clone 7E.17G9), or isotype controls. All antibodies were from BD Biosciences (San Jose, CA) unless otherwise specified. Biotin-conjugated Abs were revealed by streptavidin-PercP-Cy5a (BD Pharmingen). Foxp3 expression was determined using an intracellular Foxp3-PE staining kit (eBioscience, San Diego, CA). Cell staining was analyzed using a FACScalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). At least 30,000 events were acquired per sample, and dead cells were excluded based on scatter characteristics. Positive staining was determined as equal to or greater than the top 5% of the isotype control.

Suppression Assays

$CD4^-$ (APC), $CD4^+ CD25^-$ Teff, and $CD4^+ CD25^+$ Treg populations were purified from splenocytes with magnetic beads using the $CD4^+ CD25^+$ Treg cell kit according to the manufacturer's instructions (Miltenyi Biotec). FACS analysis of Tregs and Teffs consistently showed >90% purity. *In vitro* suppression assays were performed as previously described (189)

in the presence of 1μg/ml anti-CD3. The number of Teffs was kept constant at 5×10^5 cells/well, whereas the number of Tregs was titrated using 4-fold dilutions. To assess the suppressive function of Tregs *in vivo,* 4×10^5 $CD4^+$ $CD25^-$ Teff and $CD4^+$ $CD25^+$ Treg populations from 6 month old male donor mice were transferred into 2 month old male B6.$Rag^{-/-}$ mice in the presence or absence of 1×10^5 Tregs as previously described (189). After injection, mice were monitored for clinical signs of colitis for up to 8 weeks and body weight was monitored weekly. Mice that lost 15% or more of body weight or showed overt clinical signs of disease were sacrificed.

Bone Marrow Chimeras

Chimeras were prepared as previously described (157). In brief, 6-8 week old female B6 mice were lethally irradiated with two doses of 525 Rad γ irradiation (4 hours apart) in a Gammacell 40 ^{137}Cs apparatus (MDS Nordion). Donor BM cells were depleted of mature T cells using CD5 Microbeads (Miltenyi Biotech). Production of mixed BM chimeras was performed at a 1:1 ratio for the B6.*Thy1a* and B6.*Sle1a.1* or B6.*Sle1a.2* mice. Ten million cells were given to each mouse by tail vein injection. Chimeric mice were maintained for 8 weeks, and lymphocytes were analyzed by flow cytometry to evaluate T cell activation and Treg levels. Activation was measured by staining lymphocytes with CD4, CD90.1 (*Thy1a*), CD90.2 (*Thy1b*), and CD69 12 hours after stimulation with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml) in a 37°C, 5% CO_2 incubator. Treg levels were measured by staining lymphocytes with CD4, CD25, CD62L and either CD90.1 or CD90.2 prior to culture.

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 4 Software. Non-parametric tests and multiple-test corrections were used as appropriate and as indicated for each experiment. Unpaired t test statistics (one or two-tailed as indicated) were used to compare the

phenotypes of the B6.*Sle1a*, B6.*Sle1a.1* and B6.*Sle1a.2* strains with that of B6. Comparisons for BM chimeras were made with paired two-tailed Student's t tests after verification that the data were normally distributed with GraphPad Prism 4. Nonparametric Mann-Whitney U tests were used when the data were not normally distributed. Comparisons for colon and kidney pathology were made with one-way ANOVA tests. Each *in vitro* experiment was performed at least twice with reproducible results. Statistical significance was obtained when $p \leq 0.05$, and is indicated in figures as * : $p \leq 0.05$, ** : $p \leq 0.01$, and *** : $p \leq 0.001$.

Results

More than One Gene Contributes to *Sle1a* Phenotypes

We have used the three congenic recombinant strains shown in Fig. 4-1 to refine the location of the gene(s) responsible for the *Sle1a* phenotypes. The entire *Sle1a* interval is covered by the combination of the *Sle1a.1* and *Sle1a.2* intervals. There is a potential overlap between the two intervals between rs30711102 and rs31028646. In addition, the *Sle1a.2* interval extends on the telomeric end beyond the *Sle1a* interval, resulting in B6.*Sle1a* and B6.*Sle1a.2* having the B6 and NZW allele at the *Fcgr2b* gene, respectively (188). There are a number of genes included within the *Sle1a* region, as shown in Table 4-1, with the telomeric end showing a more dense set of genes. Some of the genes present within the interval D1MIT15 to D1MIT353, or the *Sle1a* interval, include *Pbx1*, encoding for pre-B cell leukemia transcription factor, *Cdca1*, known to be involved in the cell cycle, *Rgs5* and *Rgs4*, regulators of G proteins involved in angiogenesis and cardiac development, *Hsd17b7* or hydroxysteroid (17-beta) dehydrogenase 7, a steroid-converting enzyme that modulates steroid function, *Ddr2* or discoidin domain receptor 2, which aids in regulation of cell proliferation and expression of MMP-1 and MMP-2, *Uap1* or UDP N-acetylglucosamine pyrophoshorylase 1, known to be involved in metabolic processes, *Uhmk1* or

U2AF homology motif kinase 1, and *Sh2d1b* or Eat2, which binds SLAM-family receptors with high affinity.

Sle1a Requires Both *Sle1a.1* and *Sle1a.2* Expression for the Increased Level of T Cell Activation and Decreased Level of Tregs

Based upon work done by other members of the lab (13), it was revealed that *Sle1a* expression significantly enhances anti-nuclear autoAb production and that *Sle1a* increases the number of autoreactive B cells that respond to alloreactive T cell help, indicating that *Sle1a* contributes to lupus pathogenesis. However, neither of these phenotypes completely maps to either subloci, suggesting to us that both *Sle1a.1* and *Sle1a.2* are involved with the phenotypes observed for the entire *Sle1a* interval. We have previously reported that *Sle1* is associated with a significantly increased number of activated $CD4^+$ T cells as well as a decreased number of $CD4^+$ $CD25^+$ Tregs, and that this phenotype mapped to *Sle1a* and to a lesser extent to *Sle1c* (13). In addition, we have reported that *Sle1a* $CD4^+$ T cells showed a significant increase in expression of ICOS (189), a co-stimulatory molecule shown to be pivotal for T-B cell interactions and highly expressed on follicular helper T cells (167). Here we show that expression of *Sle1a.1*, but not *Sle1a.2*, leads to a significant increase in ICOS expression *ex vivo* (Fig. 4-2A). We did not observe this increase for either *Sle1a.1* or *Sle1a.2* when assessing CD69 expression (data not shown), although we have observed that expression of either *Sle1a.1* or *Sle1a.2* leads to a significantly decreased ratio of naïve to memory $CD4^+$ T cells (data not shown). The B6.*Sle1a* mice also showed significantly decreased percentages of $CD4^+$ $CD25^+$ cells expressing CD62L, indicating that this locus induced a higher proportion of recently activated cells $CD4^+$ $CD25^+$ cells as opposed to Tregs (189). However, the results shown in Fig. 4-2B indicate that this decrease in Tregs does not map to either the *Sle1a.1* or *Sle1a.2* locus. These findings were confirmed by assessment of intracellular expression of Foxp3 (Fig. 4-2C and D). Similar results

were obtained for 5-7 month old mice (data not shown). Overall, these results indicate that expression of *Sle1a.1* alone can account for the observed increase in ICOS expression on $CD4^+$ T cells, but expression of both *Sle1a.1* and *Sle1a.2* are required for the decreased *ex vivo* $Foxp3^+$ Treg compartment associated with *Sle1a*.

Sle1a.1 and *Sle1a.2* Tregs Can Support Inhibition to a Level Equivalent to B6 Tregs *In Vitro*

We have previously shown that while the *in vitro* inhibitory capacity of B6 and *Sle1a* Tregs was similar at a 1:1 Treg:Teff ratio, a significantly diminished inhibitory function was observed at both the 1:4 and 1:16 ratios for *Sle1a* Tregs (189). At this latter ratio, inhibition by *Sle1a* Tregs was no longer observed, and in some cases increased proliferation was observed with *Sle1a* Treg addition (189), as we have previously reported for B6.TC Tregs (158). This result is consistent with the $CD4^+$ $CD25^+$ population containing a smaller proportion of functional Tregs in B6.*Sle1a* than in B6 mice. Here we assessed the suppressive capacity of B6.*Sle1a.1* and B6.*Sle1a.2* Tregs in comparison to B6 in assays in which the only variable was the Treg origin, (B6, B6.*Sle1a.1* or B6.*Sle1a.2*), while all other cells were of B6 origin. As shown in Fig. 4-3, there was no major difference between the inhibitory capacity of B6, B6.*Sle1a.1* (Fig. 4-3A) and B6.*Sle1a.2* (Fig. 4-3B) Tregs at any Treg:Teff ratio, contrary to what we observed for *Sle1a* Tregs (189).

Sle1a.1 and *Sle1a.2* Expression Affects the Ability of Both DCs to Support Treg Suppression and Teffs to be Inhibited, but to a Lesser Extent than Observed for *Sle1a*

We have previously shown that expression of *Sle1a* in any of the three members of the suppression assay, namely Tregs, Teffs and APCs, significantly impacted the ability of Tregs to suppress Teff proliferation (189). Here we performed the same test to assess the consequences of either *Sle1a.1* or *Sle1a.2* expression in any of the three members of the suppression assay on Treg function (Fig. 4-4). As shown above and contrary to results for expression of *Sle1a*,

expression of either *Sle1a.1* or *Sle1a.2* in Tregs did not significantly affect Treg suppressive capacity. While we previously showed that *Sle1a* expression in the Teff compartment resulted in an increased resistance to Treg suppression in both the 1:1 and 1:4 Treg:Teff settings (189), expression of either *Sle1a.1* (Fig. 4-4A) or *Sle1a.2* (Fig. 4-4B) in Teffs resulted in significantly hindered Treg suppression only in lower Treg:Teff ratios. Finally, expression of *Sle1a* in APCs significantly prevented inhibition at all three ratios, and even induced enhanced proliferation at the 1:16 Treg:Teff ratio (189). However, expression of either *Sle1a.1* or *Sle1a.2* in APCs showed only a significant effect on Treg function at the 1:16 Treg:Teff ratio. Taken together, expression of both the *Sle1a.1* and *Sle1a.2* subloci is necessary to observe the phenotype associated with *Sle1a*, namely that expression in any one of the three members of the suppression assay significantly impacts the ability of Tregs to suppress Teff proliferation. Expression of either *Sle1a.1* or *Sle1a.2* alone, however, affects the ability of Teffs to resist Treg suppression and that of APC to mediate Treg suppression, although to a lower level that the entire *Sle1a* interval

We also assessed *in vivo* the effect of *Sle1a*, *Sle1a.1* and *Sle1a.2* expression on effector and regulatory $CD4^+$ T cell function in a rapid model of disease adapted from the experimental colitis model (169). Using this same model, we previously showed that Tregs from 3 month old female B6.*Sle1a* mice could not suppress colitis onset induced by either B6 or B6.*Sle1a* Teffs (189). In addition, the Teffs expressing *Sle1a* were resistant to suppression by both B6 and B6.*Sle1a* Tregs (189). In this case, B6.$Rag^{-/-}$ mice received 4 x 10^5 $CD4^+$ $CD25^-$ Teffs from either 6 month old B6 or congenic (B6.*Sle1a*, B6.*Sle1a.1* or B6.*Sle1a.2*) mice in the presence or absence of 1 x 10^5 $CD4^+$ $CD25^+$ Tregs from B6 or congenic (B6.*Sle1a*, B6.*Sle1a.1* or B6.*Sle1a.2*) mice. As expected, B6 Teffs induced colitis, here defined by a 15% loss in body weight, which was

abrogated by the presence of B6 Tregs (Fig. 4-5). Colon and kidney histology is pending for all three experiments. We also observed that in all three cases, the congenic-expressing Teffs were more potent inducers of colitis onset than B6 Teffs. Tregs expressing *Sle1a* (Fig 4-5A), and to a lesser extent those expressing *Sle1a.1* (Fig. 4-5B) and *Sle1a.2* (Fig 4-5C), did not suppress B6 Teff function as well as B6 Tregs, possibly due to a lower ratio of functional Tregs within the injected $CD4^+$ $CD25^+$ population. While we observed that those Teffs expressing *Sle1a* are resistant to suppression by B6 Tregs (Fig. 4-5A), we did not obtain this result for Teffs expressing either of the individual subloci (Fig. 4-5B and C). This *in vivo* experiment confirmed our previously published results showing that the *Sle1a* $CD4^+$ $CD25^+$ population is less effective at suppressing Teff functions and that *Sle1a* Teffs are resistant to Treg suppression, as well as confirming our *in vitro* findings above that the effect of the entire *Sle1a* locus requires expression of both *Sle1a.1* and *Sle1a.2*.

Sle1a.1 or _Sle1a.2_ Expression Intrinsically Affects $CD4^+$ T Cell Phenotypes

We have previously shown that although *Sle1a* expression affects multiple hematopoeitic cell compartments, *Sle1a* results in intrinsically activated $CD4^+$ T cells and its expression in non-hematopoeitic cells is not required for induction of these phenotypes (189). Here we used the same mixed bone marrow chimera approach for both subloci *Sle1a.1* and *Sle1a.2* and observed similar results to that of *Sle1a* (Fig. 4-6). The increased activation of $CD4^+$ T cells (Fig. 4-6A) and the decreased percentage of Tregs (Fig. 4-6B) were again completely reproduced by *Sle1a.1* and *Sle1a.2* bone-marrow-derived cells, and in the mixed bone marrow chimeras containing both congenic and normal $CD4^+$ T cells, only those T cells expressing *Sle1a.1* or *Sle1a.2* displayed enhanced activation and a decreased proportion of $CD62L^+$ Tregs. Interestingly, the increased level of activation and decreased level of Tregs were exaggerated to a level of significance when assessed in the lymphopenic environment of the mixed bone marrow chimera assay as compared

to unmanipulated mice as shown in Fig. 4-2. Taken together, we conclude that expression of either *Sle1a.1* or *Sle1a.2* results in T cell-intrinsic phenotypes. The abnormal phenotypes are not transferable to bystander normal T cells, excluding *Sle1a.1* or *Sle1a.2* being mediated through a soluble factor.

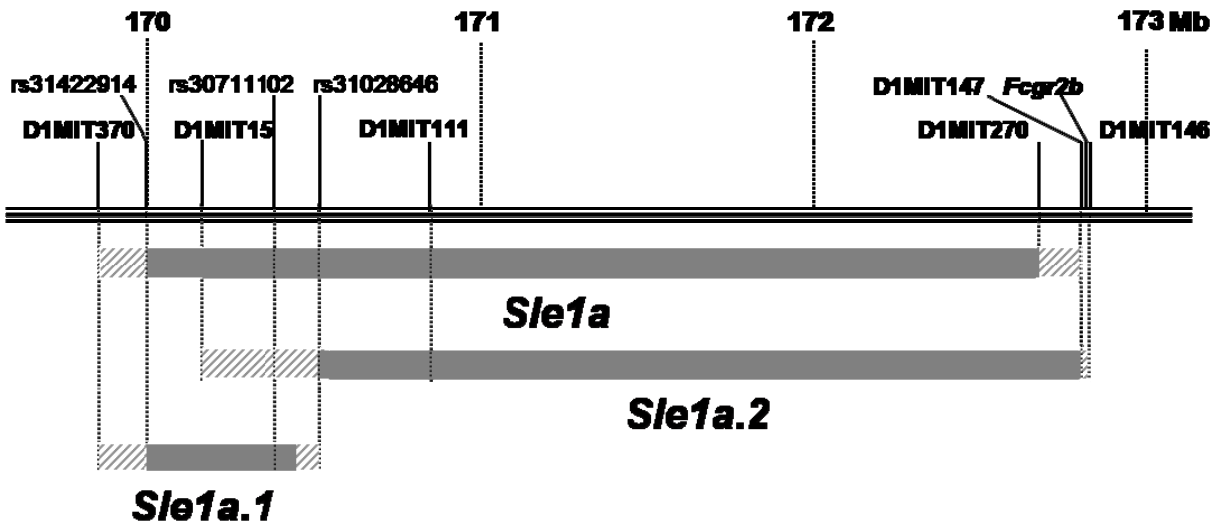

Figure 4-1. Map of *Sle1a*. From top to bottom are shown: a scale in Mb, the location of the microsatellite markers or SNPs mapping the interval termini, and the *Sle1a, Sle1a.1* and *Sle1a.2* intervals, in which the gray rectangles show the regions of known NZW allelic derivation, and the hatched rectangles on each side indicate the regions of recombination between the NZW and B6 genomes. Map based on Ensembl Release 40.

Table 4-1. Genes contained within the *Sle1a* interval according to Ensembl Release 40.

Start	End	*Sle1a* subloci	Symbol	Description
169,995,407	170,268,179	*Sle1a.1*	*Pbx1*	pre B-cell leukemia transcription factor 1
171,290,203	171,290,544	*Sle1a.2*	XP_922544.2	RefSeq peptide predicted
171,334,986	171,368,103	*Sle1a.2*	*Cdca1*	cell division cycle associated 1
171,492,176	171,532,488	*Sle1a.2*	*Rsg5*	regulator of G-protein signaling 5
171,578,985	171,584,317	*Sle1a.2*	*Rsg4*	regulator of G-protein signaling 4
171,765,614	171,771,328	*Sle1a.2*	1700084C01Rik	RIKEN cDNA
171,786,212	171,805,880	*Sle1a.2*	*Hsd17b7*	hydroxysteroid (17-beta) dehydrogenase 7
171,814,321	171,947,236	*Sle1a.2*	*Ddr2*	discoidin domain receptor family, member 2
171,978,678	172,011,621	*Sle1a.2*	*Uap1*	UDP-N-acetylglucosamine pyrophosphorylase 1
172,035,931	172,052,068	*Sle1a.2*	*Uhmk1*	U2AF homology motif (UHM) kinase 1
172,069,546	172,088,262	*Sle1a.2*	*Sh2d1c*	EAT-2-related transducer, EAT-2b
172,114,051	172,123,444	*Sle1a.2*	*Sh2d1b*	EAT-2a
172,145,478	172,148,800	*Sle1a.2*	1700015E13Rik	RIKEN cDNA
172,155,147	172,426,524	*Sle1a.2*	*Nos1ap*	nitric oxide synthase 1 (neuronal) adaptor protein
172,481,505	172,519,464	*Sle1a.2*	*Olfml2b*	olfactomedin-like 2B
172,543,889	172,704,443	*Sle1a.2*	*Atf6*	activating transcription factor 6
172,710,173	172,722,215	*Sle1a.2*	*Dusp12*	dual specificity phosphatase 12
172,743,948	172,749,816	*Sle1a.2*	*Fcrlb*	Fc receptor-like B
172,754,576	172,764,268	*Sle1a.2*	*Fcrla*	Fc receptor-like A
172,797,233	172,813,222	*Sle1a.2*	*Fcgr2b*	Fc receptor IgG low affinity IIb

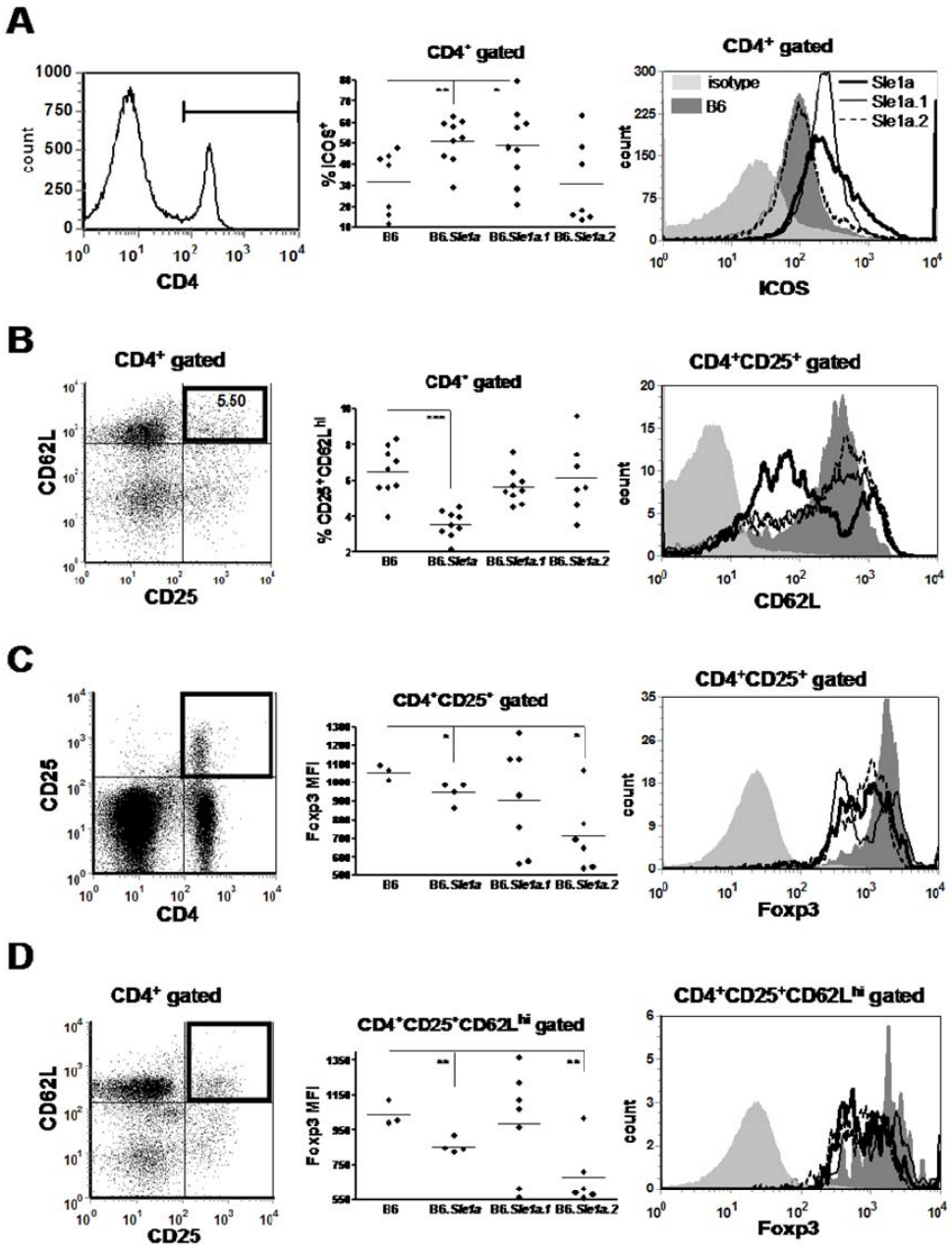

Figure 4-2. *Sle1a* requires both *Sle1a.1* and *Sle1a.2* for increased $CD4^+$ T cell ICOS expression and diminished Treg compartment. Splenocytes from B6, B6.*Sle1a*, B6.*Sle1a.1* and B6.*Sle1a.2* mice were labeled for surface CD4, ICOS (A), CD25, and CD62L (B) and intracellular Foxp3 (C-D) expression and analyzed by FACS. Each point represents an 8-12 month old individual animal. Representative gatings on a B6 sample are shown in the left-hand column (marker for A and rectangular gate for B-D) and representative histograms for all four strains are shown in the right-hand column. The light gray-filled histograms show isotype controls, dark gray-filled histograms show B6 values, while thick, thin and dashed black lines represent B6.*Sle1a*, B6.*Sle1a.1* and B6.*Sle1a.2*, respectively. All comparisons were performed with B6 values. One-tailed *t* tests: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

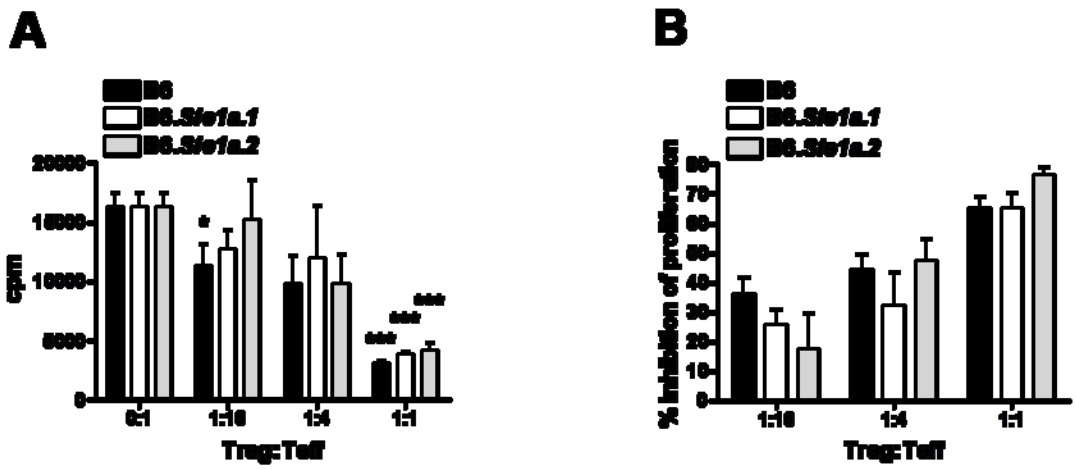

Figure 4-3. *Sle1a.1* and *Sle1a.2* Tregs can support inhibition to a level equivalent to B6 Tregs *in vitro.* Inhibition of proliferation assays were set up with B6-derived APCs and Teffs, and either B6 (black bars), B6.*Sle1a.1* (white bars) or B6.*Sle1a.2* (gray bars) Tregs at the indicated ratio. A) Representative assay comparing proliferation in the presence of B6, B6.*Sle1a.1* or B6.*Sle1a.2* Tregs (4 mice per strain). Means and SEs are results of one-tailed *t* tests between the 0:1 assays and the various Treg:Teff ratios for each strain. B) Normalized percentage inhibition of proliferation of the 0:1 assays at the various Treg:Teff ratios for each strain combined from two different assays (8 mice per strain). Means and SEs are results of one-tailed *t* tests between the three strains for each Treg:Teff ratio. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

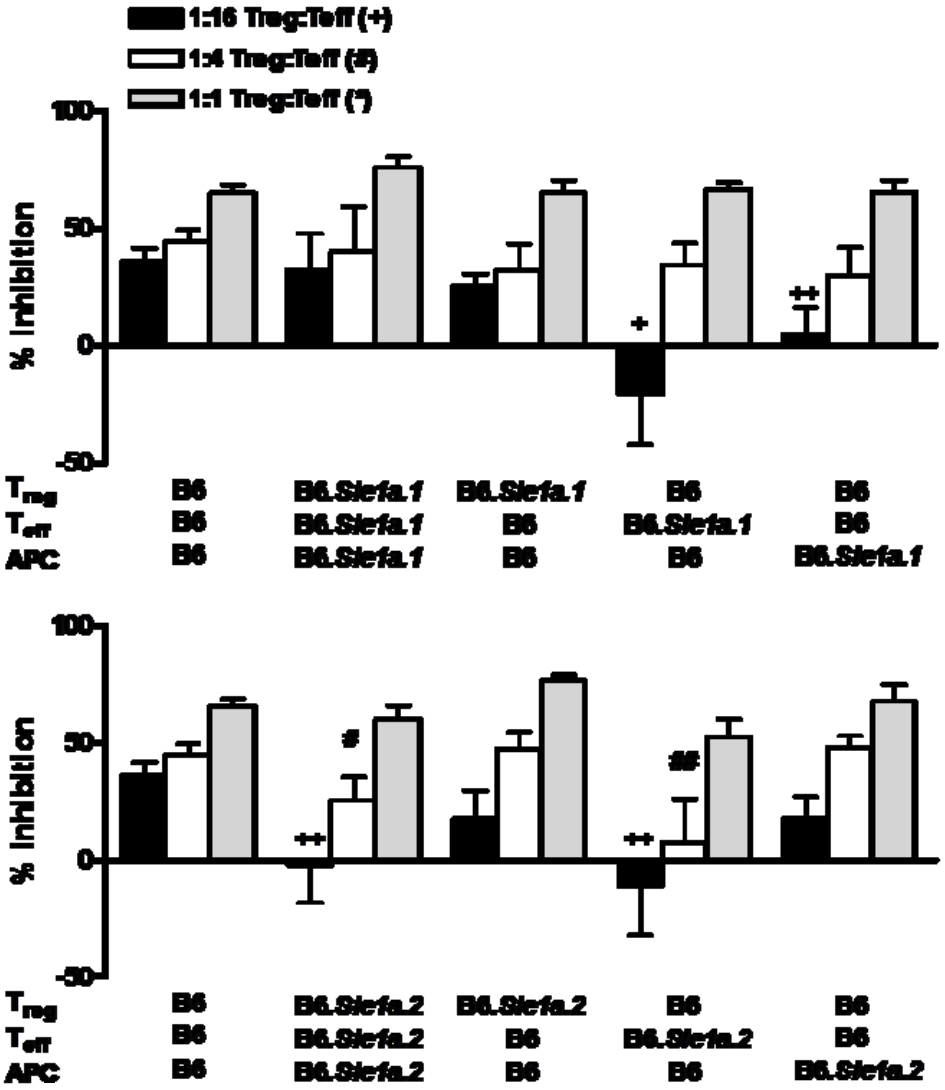

Figure 4-4. *Sle1a.1* or *Sle1a.2* expression in Teffs or APCs affects the extent of the inhibition of Teff proliferation. The inhibition of $CD4^+$ $CD25^+$ Teff proliferation in presence of 1:1, 1:4, or 1:16 Treg:Teff ratios is expressed as a percentage of the proliferation induced in the absence of Tregs for each condition. The origin, B6, B6.*Sle1a* (A), or B6.*Sle1a.2* (B) of Tregs, Teffs, and APCs is indicated under each column. The graphs show the means and standard errors of two independent assays with 3-4 mice per strain, age 6 months in each assay. Results of one-tailed *t* tests between each condition and the "all B6 condition" are indicated for each Treg:Teff ratio. $^{+,\#,}$ *: $p<0.05$, $^{++,\#\#,}$ **: $p<0.01$, $^{+++,\#\#\#,}$ ***: $p<0.001$.

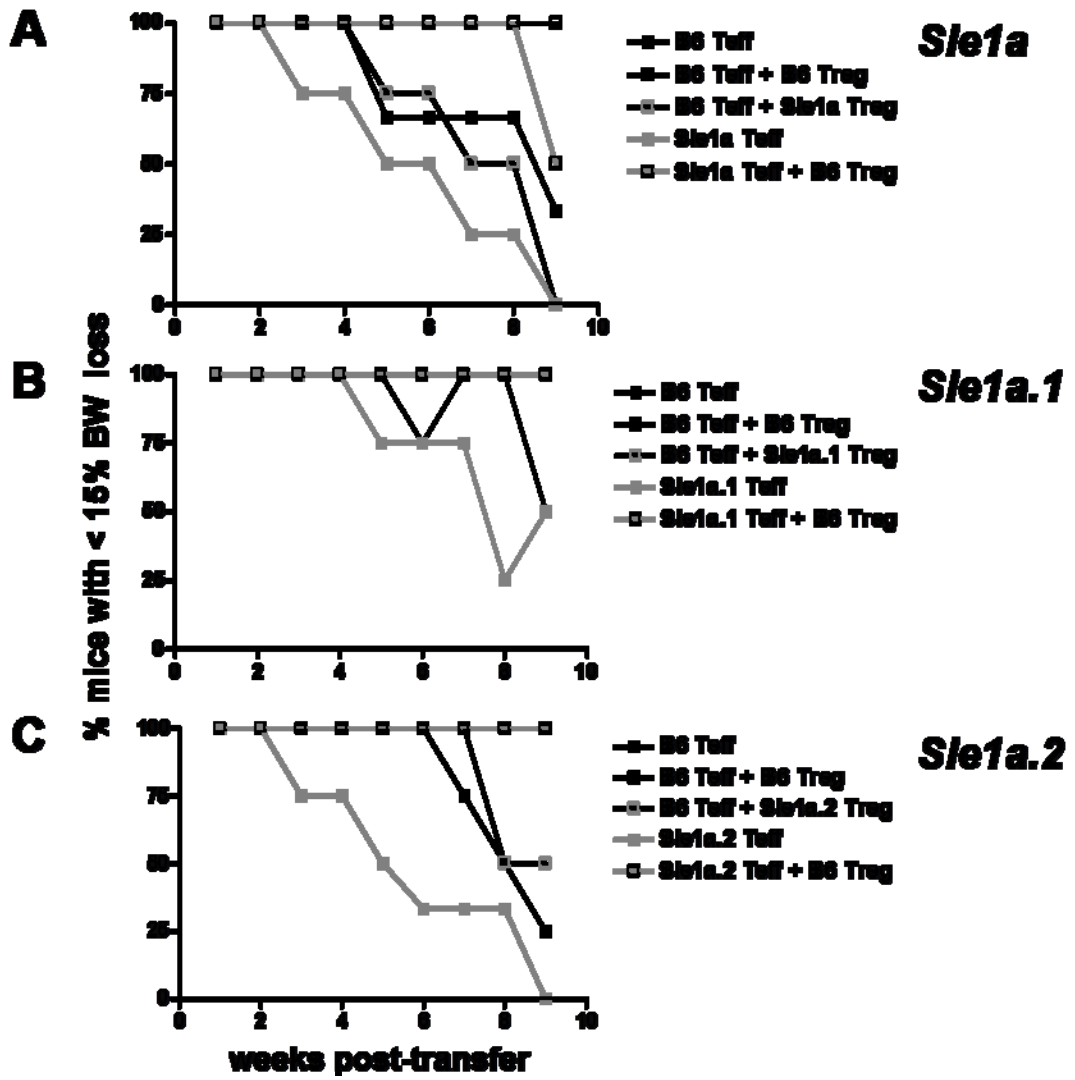

Figure 4-5. Expression of *Sle1a*, *Sle1a.1* or *Sle1a.2* in either Tregs or Teffs affects the extent of disease in an adoptive transfer model. $CD4^+$ $CD25^-$ Teff and $CD4^+$ $CD25^+$ Treg populations from 6 month old male donor mice were transferred by tail-vain injection into 2 month old male B6.$Rag1^{-/-}$ recipient mice. Lines are representative of the percentage of mice per group (n=4) with less than 15% body weight (BW) loss over a 10 week period post-injection. Individual experiments for *Sle1a* (A), *Sle1a.1* (B), and *Sle1a.2* (C) donors are shown. Line depictions are as follows: B6 Teffs only - black line / black filled box, congenic Teffs only - gray line / gray filled box, B6 Tregs:B6 Teffs - black line / black open box, B6 Teffs:congenic Tregs - black line / gray open box, B6 Tregs:congenic Teffs - gray line / black open box. Colon and kidney histology is pending for all three experiments.

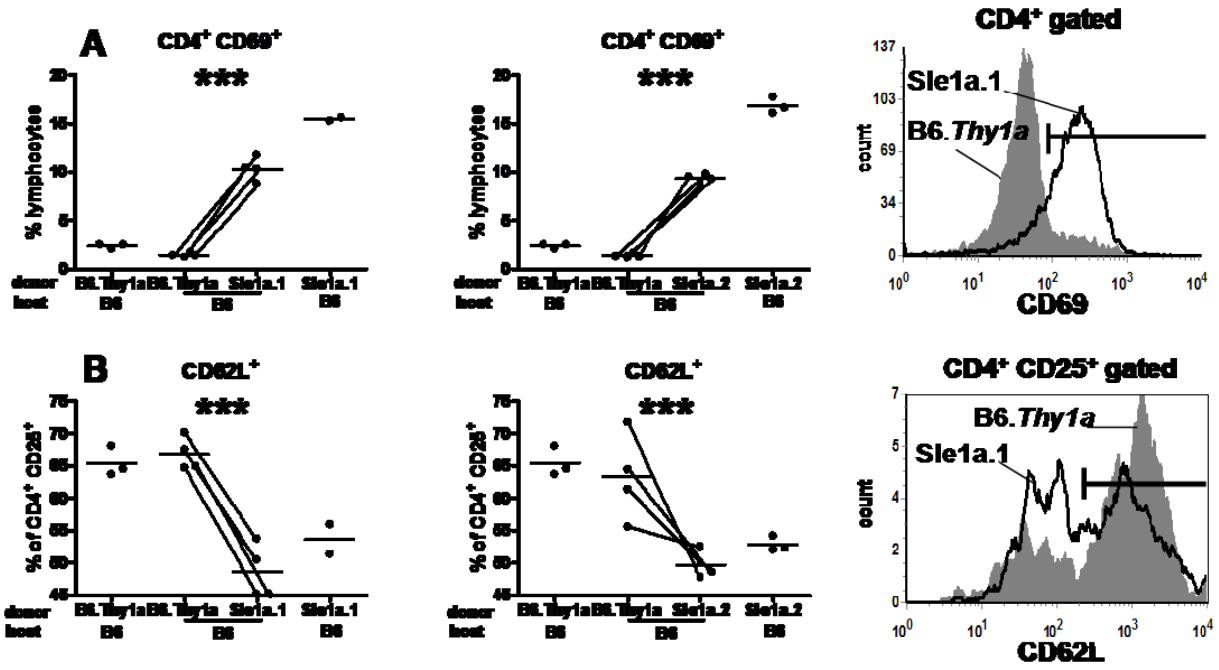

Figure 4-6. Expression of either *Sle1a.1* or *Sle1a.2* affects $CD4^+$ function in a cell-intrinsic manner. B6 hosts were reconstituted with B6.*Thy1a* and/or B6.*Sle1a* subloci (*Thy1b* allele) bone marrow. Connected samples indicate values for $CD4^+$ T cells expressing the *Thy1a* (CD90.1-gated) or *Thy1b* (CD90.2-gated) alleles within the same mouse. Controls are represented by B6.*Thy1a*→B6 and B6.*Sle1a.1* or B6.*Sle1a.2*→B6 single-strain bone marrow transfers. A) Activation measured as the percentage of $CD4^+$ $CD69^+$ lymphocytes with representative histogram showing CD69 expression on gated $CD4^+$ T cells. B) Treg levels, measured as the percentage of $CD4^+$ $CD25^+$ splenocytes expressing CD62L with representative histogram showing CD62L expression. Each point represents an individual animal. Two-tailed *t* tests: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

CHAPTER 5
MURINE LUPUS SUSCEPTIBILITY LOCUS *SLE1A.1* CONTROLS RETINOIC ACID-ENHANCED TGF-BETA-INDUCED REGULATORY T CELL EXPANSION

Introduction

In the previous chapter, we presented results which indicated that the phenotypes resulting from expression of *Sle1a* require the synergistic effect of both of its subloci, *Sle1a.1* and *Sle1a.2*, due to the intermediate phenotypes observed for each. From this, we can infer that both subloci contain genes necessary for SLE susceptibility, and can now progress to the assessment of which genes are directly involved since the regions of susceptibility have now been narrowed. We will now focus on lupus susceptibility locus *Sle1a.1* due to its small size of less than 1 Mb.

Materials and Methods

Mice

B6.*Sle1a.1*$^{NZW/NZW}$ (B6.*Sle1a.1*) was obtained as a recombinant interval from B6.*Sle1a* (Fig. 4-1). C57BL/6J (B6) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME).). B6.FOXP3-eGFP mice were obtained from the Kuchroo group and derived as described (190). We derived the B6.*Sle1a.1*.FOXP3-eGFP mice by breeding our B6.*Sle1a.1* to Kuchroo's B6.FOXP3-eGFP mice using standard congenic breeding techniques. All mice were bred and maintained at the University of Florida in specific pathogen-free conditions. All experiments were conducted according to protocols approved by the University of Florida Institutional Animal Care and Use Committee.

Sle1a.1 Map and *Pbx1* Primers

PCR genotyping with microsatellite markers was performed as previously described (12) and SNP genotyping was performed by direct sequencing. Genes were found using Ensembl Release 40 (www.ensembl.org). There are 10 exons associated with Pbx1. Primers were designed around exons 5-8, in order to visualize isoform Pbx1-a from Pbx1-b, as Pbx1-b lacks

exon 7, which does not appear to affect its function. Our collaborator, Dr. Shiwu Li designed the following primers, 5' - GAA GTG CGG CAT CAC AGT CTC- 3' from exon 5 and 5' - ACT GTA CAT CTG ACT GGC TGC - 3' from exon 8, to assess expression levels of multiple isoforms of Pbx1. $CD4^+$ T cells from 5 month old female B6 and B6.*Sle1a.1* mice were isolated by negative selection using the SpinSep for Mouse $CD4^+$ T cell enrichment protocol (Stemcell Technologies). B cells from the same mice were isolated by negative selection using the B cell purification kit from Miltenyi Biotec. FACS analysis of the resulting $CD4^+$ and B cell populations consistently showed >90% purity.

Flow Cytometry

Briefly, cells were first blocked on ice with staining buffer (PBS, 5% horse serum, and 0.09% sodium azide) supplemented with 10% rabbit serum and pretreated with anti-CD16/CD32 (2.4G2). Cells were then stained with a pre-titrated amount of PE-conjugated CD4 (RM4–5) from BD Biosciences (San Jose, CA). Foxp3 expression was determined by visualizing GFP expression. Cell staining was analyzed using a FACScalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). At least 30,000 events were acquired per sample, and dead cells were excluded based on scatter characteristics. Positive staining was determined as equal to or greater than the top 5% of the isotype control.

Apoptotic Cell-Induced Production of IL-10 by Peritoneal Macrophages

Peritoneal macrophages were obtained by injection of 2.5 ml of thioglycollate medium (3%) intraperitoneally into female B6 and B6.*Sle1a.1* mice at 6 months of age (n=4). Macrophages were harvested 4 days later by peritoneal lavage. Induction of apoptosis was as described previously (191). Briefly, female B6 thymocytes at a concentration of 2×10^6 cells/ml were cultured with 15 μg/ml staurosporine (Cayman Chemical, Ann Arbor, MI) for 8 hours at 37°C in 5% CO_2 to induce a population of early-apoptotic cells. The percentage of early

apoptotic cells was quantified by flow cytometry analysis by using Annexin-V and 7-AAD staining according to the manufacturer's instructions and was routinely 50-60%. These apoptotic cells were then cultured, according to a previous protocol (150), with the thioglycollate-induced peritoneal macrophages elicited from male B6 and B6.*Sle1a.1* mice at a ratio of 2:1 apoptotic cells to macrophages for 12 hours at 37°C in 5% CO_2 and analyzed for IL-10 production in the culture supernatant using an OptEIA Mouse IL-10 ELISA kit (BD Pharmingen) according to manufacturer's instructions.

Microarray Analysis for Differential Gene Expression

$CD4^+$ T cells from 5 month old female B6 and B6.*Sle1a.1* mice were isolated by negative selection using the SpinSep for Mouse $CD4^+$ T cell enrichment protocol (Stemcell Technologies). FACS analysis of the resulting $CD4^+$ population consistently showed >90% purity. Our collaborator, Dr. Zhiwei Xu used the RNeasy Mini Kit (Qiagen) to isolate RNA from 3 x 10^6 B6 and B6.*Sle1a.1* $CD4^+$ T cells. cDNA was made, amplified, and labeled with biotin to prepare for hybridization to the Affymetrix Mouse Genome 430 2.0 Array using the Ovation Biotin RNA Amplification and Labeling System (NuGEN Technologies, Inc.). Microarray data obtained were normalized with the Affymetrix Microarray Suite (MAS 5.0), based on the housekeeping gene expression profile. Expression values were adjusted to the intensity of the expression value of the 100 housekeeping genes. Supervised analysis was performed by Dr. Henry Baker with a $p < 0.001$. Dr. Igor Dozmorov further analyzed the microarray data using the following methods. Identification of differentially expressed genes was carried out with use associative analysis presented elsewhere (192). Cross-validation of the selections involved using a jacknife procedure for characterization of the robustness or reproducibility (R) of the differentially expressed genes selection. The comparative analysis was repeated for the two groups of samples with exclusion every time of one sample from each

group. For two groups with *n* and *m* replicates, there is *n* x *m* possible comparisons. Genes selected as differentially expressed in each of these comparisons (selected *n* x *m* times) were ranked as having 100% of reproducibility. Analysis of associations of the obtained selections with known biologically important signaling pathways and molecules was carried out by Ingenuity© Pathway Analysis (IPA)-web-based programs (www.ingenuity.com).

Induction of $CD4^+$ $Foxp3^+$ Tregs from $CD4^+$ $CD25^-$ T Cells

$CD4^+$ $CD25^-$ cells were purified from 3 month old female B6 and B6.*Sle1a.1* splenocytes with magnetic beads using the $CD4^+$ $CD25^+$ Treg cell kit according to the manufacturer's instructions (Miltenyi Biotec). $CD4^+$ $CD25^-$ cells (5 x 10^5 cells/ml) from both B6 and B6.*Sle1a.1* mice were stimulated with 1 μg/ml anti-CD3 and 10 μg/ml anti-CD28 plate-bound antibody in the presence of 100 U IL-2 (Peprotech), 20 ng/ml TGF-β (Peprotech), and 5 nM RA (Sigma-Aldrich) and cultured at 37°C in 5% CO_2 for 5 days. Cell were then labeled for surface CD4 and intracellular Foxp3 expression and analyzed by fluorescence-activated cell sorting.

Results

***Sle1a.1* Contains Only One Gene**

We have used the three congenic recombinant strains shown in Fig. 5-1 to refine the location of the gene(s) responsible for the *Sle1a* phenotypes. The entire *Sle1a* interval is covered by the combination of the *Sle1a.1* and *Sle1a.2* intervals. There is a short overlap between the two intervals between rs30711102 and rs31028646. In addition, the *Sle1a.2* interval extends on the telomeric end beyond the *Sle1a* interval, resulting in B6.*Sle1a* and B6.*Sle1a.2* having the B6 and NZW allele at the *Fcgr2b* gene, respectively (188). Based on information retrieved from Ensembl Release 40 (www.ensembl.org), there is only one gene present in the *Sle1a.1* locus, *Pbx1*, which encodes for pre-B cell leukemia transcription factor (Pbx1), and this gene is not present in the *Sle1a.2* region (Fig. 5-1).

Sle1a.1 Expression Leads to a Decreased Ratio of $CD4^+$ $Foxp3^+$ to Total $CD4^+$ T Cells

B6.FOXP3-eGFP mice were obtained from the Kuchroo group and derived as described (190). These mice express GFP whenever Foxp3 is expressed, and are a useful tool to analyze the Treg population. The B6.*Sle1a.1*.FOXP3-eGFP mice were derived by breeding our B6.*Sle1a.1* mice to Kuchroo's B6.FOXP3-eGFP mice using standard congenic breeding techniques in order to more accurately study the Treg population in the context of expression of the NZW allele of *Pbx1*. Splenocytes from 3 month old B6.FOXP3-eGFP and B6.*Sle1a.1*.FOXP3-eGFP mice were labeled for surface CD4 expression and analyzed by fluorescence-activated cell sorting. As shown in Fig. 5-2, there is a significantly decreased ratio of $CD4^+$ $Foxp3^+$ T cells to total $CD4^+$ T cells in the B6.*Sle1a.1*.FOXP3-eGFP mice as compared to the B6.FOXP3-eGFP mice. Interestingly, by analyzing the Treg population in this fashion, we reached a level of significance, indicating that the presence of activated cells within the Treg population of the B6.*Sle1a* mice was indeed a confounding factor since we did not see this level of significance for the Treg population as defined by expression of CD4, CD25 and CD62L in Fig. 4-1. Further analysis is in the process of being completed due to the small number of samples we currently have as well as the fact that these mice are much younger compared to the 8-12 month old mice we analyzed in Fig. 4-1.

Alternative Pbx1 Isoforms

Since *Pbx1* was the only gene present in the *Sle1a.1* locus, we needed to determine how the NZW allele of this gene differed from the B6 allele. Both sequence analysis at the cDNA level and preliminary Affymetrix microarray data for expression of *Pbx1* obtained from $CD4^+$ T cells revealed no difference between the B6 and NZW allele of *Pbx1*. We wanted to assess the expression level of Pbx1 in B cells, as it has already been described as a necessary factor in very early B cell commitment (148) as well as $CD4^+$ T cells, since we have previously shown in

Chapter 4 that expression of *Sle1a.1* results in $CD4^+$ T cell-intrinsic defects. There are 10 exons associated with Pbx1. Primers were designed by our collaborator, Dr. Shiwu Li, that would be capable of detecting both of the known isoforms of Pbx1, Pbx1-a and Pbx1-b. Primers were designed around exons 5-8, in order to distinguish isoform Pbx1-a from Pbx1-b, as Pbx1-b lacks exon 7, which does not appear to affect its function. Interestingly, using these primers, we found two novel isoforms. Sequence analysis was performed on all bands to confirm that that correspond to the inferred Pbx1 isoforms based on size. Fig. 5-3 shows PCR products using primers designed for *Pbx1*, with corresponding exon / intron structure shown in Fig.5-4. Known isoforms Pbx1-a and Pbx1-b, 391 bp and 278 bp, respectively, are only found in B cells of both strains. Two novel isoforms were observed, Pbx1-c, 167 bp, found in both B and $CD4^+$ T cells of both strains and Pbx1-d, 118 bp, found in both B and T cells, but only in the B6.*Sle1a.1* strain. Pbx1-c lacks exon 7 and and only a portion of exon 6, while Pbx1-d lacks both exons 6 and 7 entirely (Fig 5-4). Pbx1 contains the following domains: the meis binding domain (MIM), the nuclear localization signal (NLS), the hox binding domain (HCM), the PBC homeodomain (PBC-A,B), and the homeo DNA binding domain (HD). Based on sequence analysis, Pbx1-c appears to lack the HCM, potentially rendering it incapable of binding Hox, while Pbx1-d appears to lack both the HCM and the HD, rendering this isoform incapable of binding both Hox and DNA. Since this isoform presumably cannot bind DNA, we predicted that its function would be impaired in the cells in which it is expressed.

Expression of *Sle1a.1* did not Affect Apoptotic Cell-Induced Production of IL-10 by Macrophages

Based on a study done by the Ma group and published in 2007, Pbx1, and more specifically Pbx1-b, was shown to be a physiologically critical mediator of apoptotic cell-induced IL-10 gene transcription and IL-10 cytokine production by macrophages , with its

transcriptional role found to be uncoupled from phagocytosis (150). Because expression of the NZW allele of *Pbx1* leads to the presence of alternative Pbx1 isoforms in immune cells, we hypothesized that the *Sle1a.1*-expressing macrophages would produce less IL-10 in response to stimulation with apoptotic cells. Thymocytes were isolated from B6 mice and stimulated with staurosporine, a potent inhibitor of protein kinases known to induce apoptosis, and subsequently cultured for 12 hours with thioglycollate-elicited peritoneal macrophages from B6 and B6.*Sle1a.1* mice. IL-10 production by the macrophages in the culture supernatant was measured by ELISA. As shown in Fig. 5-5, we found no difference between the normal and *Sle1a.1*-expressing macrophages regarding IL-10 production in response to apoptotic cells. Our collaborator, Dr. Li, assessed which isoforms of Pbx1 were present in both B6 and B6.*Sle1a.1*-derived peritoneal macrophages and found that macrophages from B6 mice expressed only Pbx1-b while macrophages from B6.*Sle1a.1* mice expressed Pbx1-a and Pbx1-b, with more of the Pbx1-b isoform present. We concluded that since both of these isoforms are capable of normal function, the production of IL-10 by macrophages from either strain would be normal as well. Our results confirmed this hypothesis.

Microarray Analysis of Differential Gene Expression Influenced by the NZW allele of *Pbx1*

The NZW allele of *Pbx1* led to alternative and novel isoforms of the transcription factor, and it was of interest to find out the effect of this alteration in a more global sense. By subjecting cDNA prepared from B6 and B6.*Sle1a.1* $CD4^+$ T cells to Affymetrix microarray hybridization and analysis and then evaluating differential gene expression, we were able to gain a better understanding of how the NZW allele of *Pbx1* globally affected this cell population. The Affymetrix Mouse Genome 430 2.0 Array is the most comprehensive whole mouse genome expression array, with analysis of over 39,000 transcripts on a single array. Table 5-1 shows a partial list of differentially expressed genes with a confidence level of greater than 50 that the

results are robust and reproducible, labeled as the R value. Of the first 80 genes that were found to be differentially expressed between B6 and B6.*Sle1a.1* $CD4^+$ T cells, 16 were involved in retinoic acid signaling. Fig 5-6 shows a heat map based on the expression of 14 of these genes known to be regulated by retinoic acid. The B6.*Sle1a.1* $CD4^+$ T cells appear to have an overall lower expression of this set of genes involved in retinoic acid signaling compared to B6 $CD4^+$ T cells. Analysis of associations of the obtained selections with known biologically important signaling pathways and molecules was carried out by Ingenuity© Pathway Analysis (IPA) - web-based programs by our collaborator, Dr. Igor Dozmorov. As shown in Fig. 5-7, the RA-signaling pathway potentially has a direct effect on Pbx1. By examining clusters of distinct yet integrated pathways, we were able to form the hypothesis that the NZW allele of *Pbx1* may lead to a difference in the capability of the resulting isoform of Pbx1, Pbx1-d, to function as a mediator of RA-signaling.

Expression of *Sle1a.1* Results in Defective RA-Enhanced TGF-β-Induced Production of Adaptive Tregs

We have presented that the NZW allele of the *Pbx1* gene leads to an alternative isoform of the Pbx1 transcription factor that is predicted to lack the DNA binding domain, presumably rendering the protein nonfunctional thereby leading to a dominant negative mutation. $CD4^+$ T cells from the B6.*Sle1a.1* mice which possess this potentially nonfunctional isoform of Pbx1 were shown to have differential expression of genes involved in the RA-signaling pathway. Since both the Noelle and Kuchroo groups previously showed that RA can enhance TGF-β-induced production of aTregs (127,190), we wanted to assess whether the $CD4^+$ $CD25^-$ T cells from the B6.*Sle1a.1* mice reacted differently to treatment with RA than normal B6 $CD4^+$ $CD25^-$ T cells. $CD4^+$ $CD25^-$ T cells isolated from both B6 and B6.*Sle1a.1* mice were cultured with anti-CD3, anti-CD28, and IL-2 in the presence or absence of TGF-β ± RA for 5 days and then stained

for surface CD4 and intracellular Foxp3 expression. We observed no Foxp3 induction in the presence of only IL-2 and there was no difference between B6 and B6.*Sle1a.1* in the percentage of $CD4^+$ $Foxp3^+$ cells when TGF-β was present (Fig. 5-8A). RA alone cannot induce Foxp3 expression in the presence of IL-2, but when TGF-β is present, we see an increase in the $CD4^+$ $Foxp3^+$ population for B6, with B6.*Sle1a.1* not showing as substantial an increase. Figs. 5-8B and C show the significant decrease in both the percent increase (Fig 5-8B) and fold increase (Fig. 5-8C) values with RA addition for the *Sle1a.1*-expressing cells, indicating that there is a defect in the RA signaling pathway that participates in TGF-β-induced Foxp3 expression. We believe this is mediated by Pbx1, and we predict that the potential lack of DNA binding by the Pbx1-d isoform contributes to this defect in RA-enhanced TGF-β-induced production of aTregs.

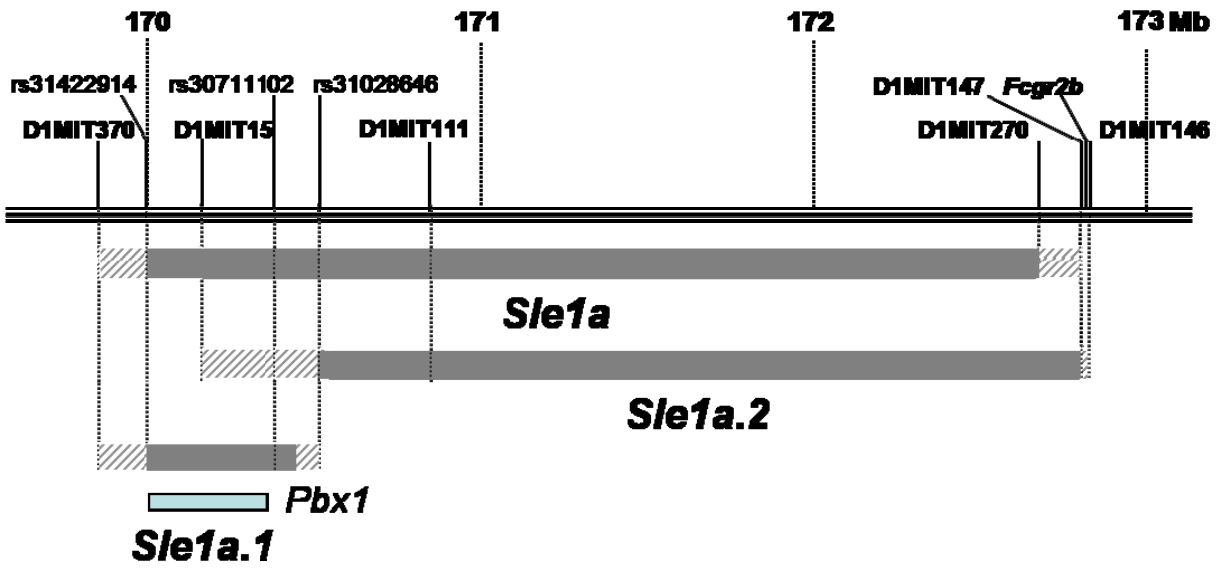

Figure 5-1. Genes present in the *Sle1a.1* interval. From top to bottom are shown: a scale in Mb, the location of the microsatellite markers or SNPs mapping the interval termini, and the *Sle1a, Sle1a.1* and *Sle1a.2* intervals, in which the gray rectangles show the regions of known NZW allelic derivation, and the hatched rectangles on each side indicate the regions of recombination between the NZW and B6 genomes. Map based on Ensemble Release 40. Pale blue box indicates the only gene present within the *Sle1a.1* region.

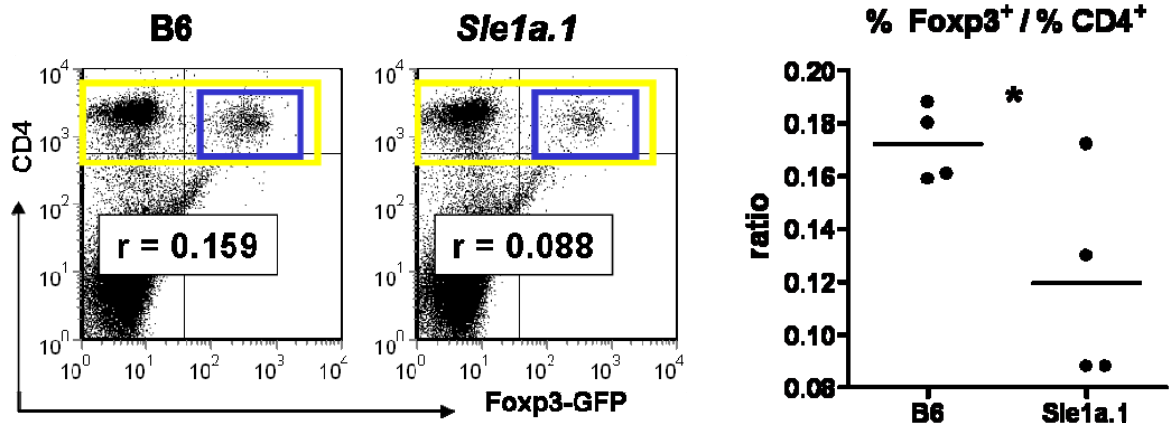

Figure 5-2. *Sle1a.1* expression results in a decreased ratio of $CD4^+$ $Foxp3^+$ to total $CD4^+$ T cells. Splenocytes from 3 month old B6.FOXP3-eGFP and B6.*Sle1a.1*.FOXP3-eGFP mice were labeled for surface CD4 expression and analyzed by fluorescence-activated cell sorting. Each point represents an individual animal. Representative plots for CD4 and GFP expression are shown on the left. Ratios (r) are derived from the % $CD4^+$ GFP^+ T cells (blue rectangle) divided by the % total $CD4^+$ T cells (yellow rectangle) and are shown on the right. Two-tailed *t* test: *: $p<0.05$.

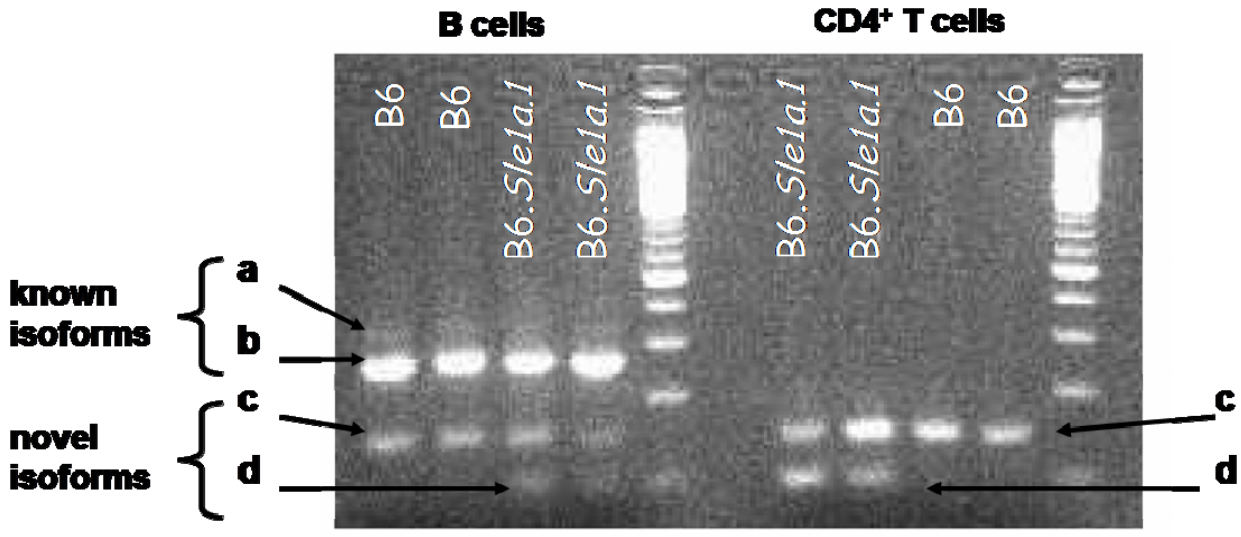

Figure 5-3. The NZW allele of *Pbx1* results in a two novel isoforms. PCR was performed on B cells and $CD4^+$ T cells isolated from 5 month old female B6 and B6.*Sle1a.1* mice using Pbx1 primers designed by Dr. Shiwu Li. Known isoforms Pbx1-a, 391 bp, and Pbx1-b, 278 bp, and are only found in B cells of both strains. Two novel isoforms were observed, Pbx1-c, 167 bp, found in both B and $CD4^+$ T cells for both strains and Pbx1-d, 118 bp, found in both B and T cells, but only in the B6.*Sle1a.1* strain.

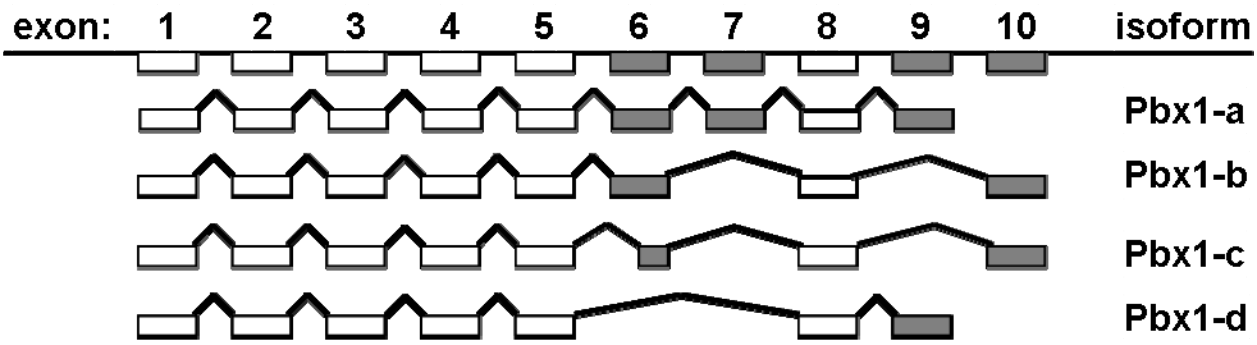

Figure 5-4. Depiction of exons associated with both known and novel isoforms of Pbx1. Primers were designed by our collaborator for *Pbx1* around exons 5-8 to be able to visualize the known isoforms, Pbx1-a and Pbx1-b, as Pbx1-b lacks exon 7. Two novel isoforms were observed using these primers and sequence analysis was done to confirm that Pbx1-c and Pbx1-d contained the exons shown above. Gray boxes indicate exons potentially not contained or different among the known and novel isoforms.

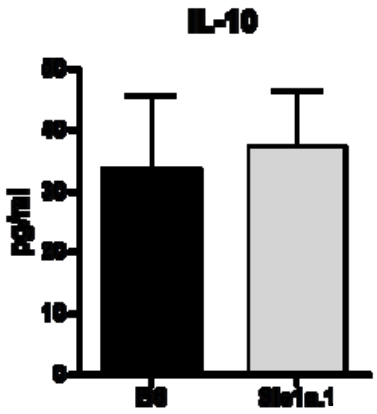

Figure 5-5. Expression of the NZW allele of *Pbx1* in macrophages does not alter their IL-10 production in response to apoptotic cells. A total of 0.5 x 10^5 B6 or B6.*Sle1a.1* thioglycollate-elicited peritoneal macrophages were stimulated with B6-derived apoptotic cells at a 2:1 ratio of apoptotic cells to macrophages for 12 hours and analyzed for IL-10 production. Graph is representative of three experiments with 4 mice per group at 6 months of age.

Table 5-1. Microarray data obtained from B6 and B6.*Sle1a.1*-derived $CD4^+$ T cells.

		Strain				
		B6		B6.*Sle1a.1*		
Affymetrix ID	Gene	Mean	SD	Mean	SD	R
1436134_at	*Scn2b*	39	10	0	0	100
1442946_at	*Atxn7*	48	12	2	0	100
1438855_x_at	*Tnfaip2*	155	36	58	12	100
1451608_a_at	*Tspan33*	57	5	24	10	100
1416105_at	*Nnt*	58	15	20	4	100
1416708_a_at	*Gramd1a*	51	3	20	1	100
1419839_x_at	*Prpf19*	117	27	56	11	100
1437614_x_at	*Zdhhc14*	61	14	23	7	100
1438711_at	*Pklr*	77	12	34	9	100
1449635_at	*Prpf19*	114	25	52	12	100
1451453_at	*Dapk2*	65	20	14	3	100
1418634_at	*Notch1*	26	10	76	19	100
1427301_at	*cd48*	31	15	83	12	100
1456103_at	*Pml*	57	21	17	6	86
1434184_s_at	*Map4k4*	36	14	85	19	86
1450543_at	*Myo1h*	38	10	83	10	86
1423389_at	*Smad7*	28	13	65	12	86
1435912_at	*Ubxd7*	29	6	66	12	86
1424631_a_at	*Ighg*	16	3	140	108	86
1416326_at	*Crip1*	36	10	71	18	86
1429184_at	*Gvin1*	122	20	225	26	86
1444003_at	*Lincr*	62	12	30	9	71
1435679_at	*Optn*	77	18	38	8	71
1436847_s_at	*Cdca8*	49	10	21	1	71
1428842_a_at	*Ngfrap1*	59	7	31	6	71
1435930_at	*Zfp291*	85	9	43	8	71
1419406_a_at	*Bcl11a*	71	27	37	5	71
1428942_at	*mt2*	72	34	16	5	71
1433761_at	*Pde4dip*	42	18	98	22	71
1418970_a_at	*Bcl10*	52	18	116	19	57
1424227_at	*Polr3h*	58	13	29	3	57
1417621_at	*Nfatc1*	71	23	138	34	57
1436886_x_at	*Xab2*	55	11	28	4	57
1437142_a_at	*Pigo*	83	18	43	7	57
1417376_a_at	*Cadm1*	64	23	32	6	57
1436934_s_at	*Aco2*	87	26	43	6	57
1457669_x_at	*Rfc2*	71	23	30	7	57
1430029_a_at	*Tspan31*	34	10	84	25	57

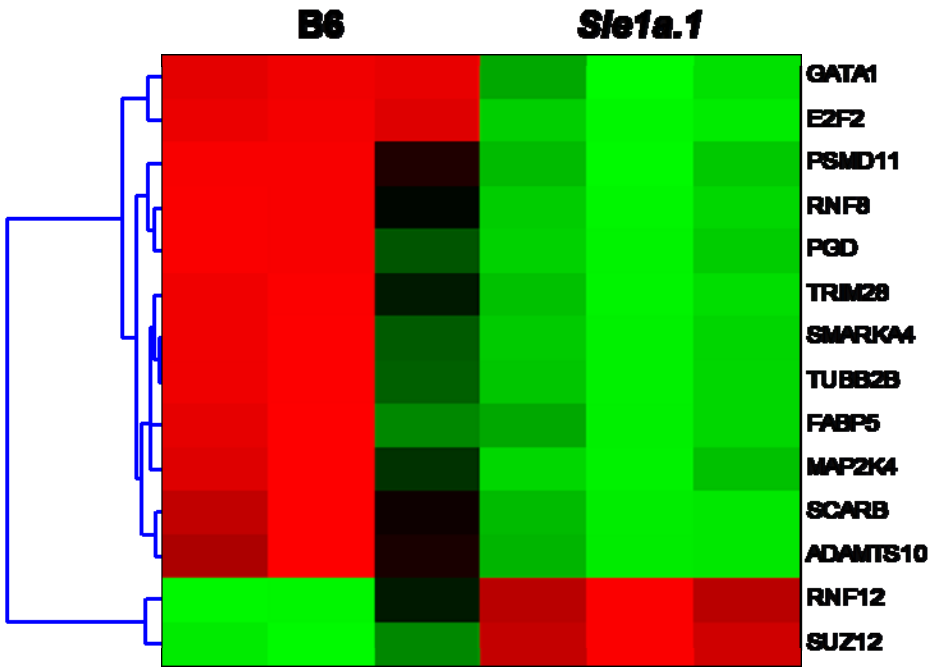

Figure 5-6. Expression of the NZW allele of *Pbx1* in $CD4^+$ T cells leads to differentially expressed genes involved in the RA-signaling pathway. Heat map based on Affymetrix microarray data from the B6 and B6.*Sle1a.1*-derived $CD4^+$ T cells.

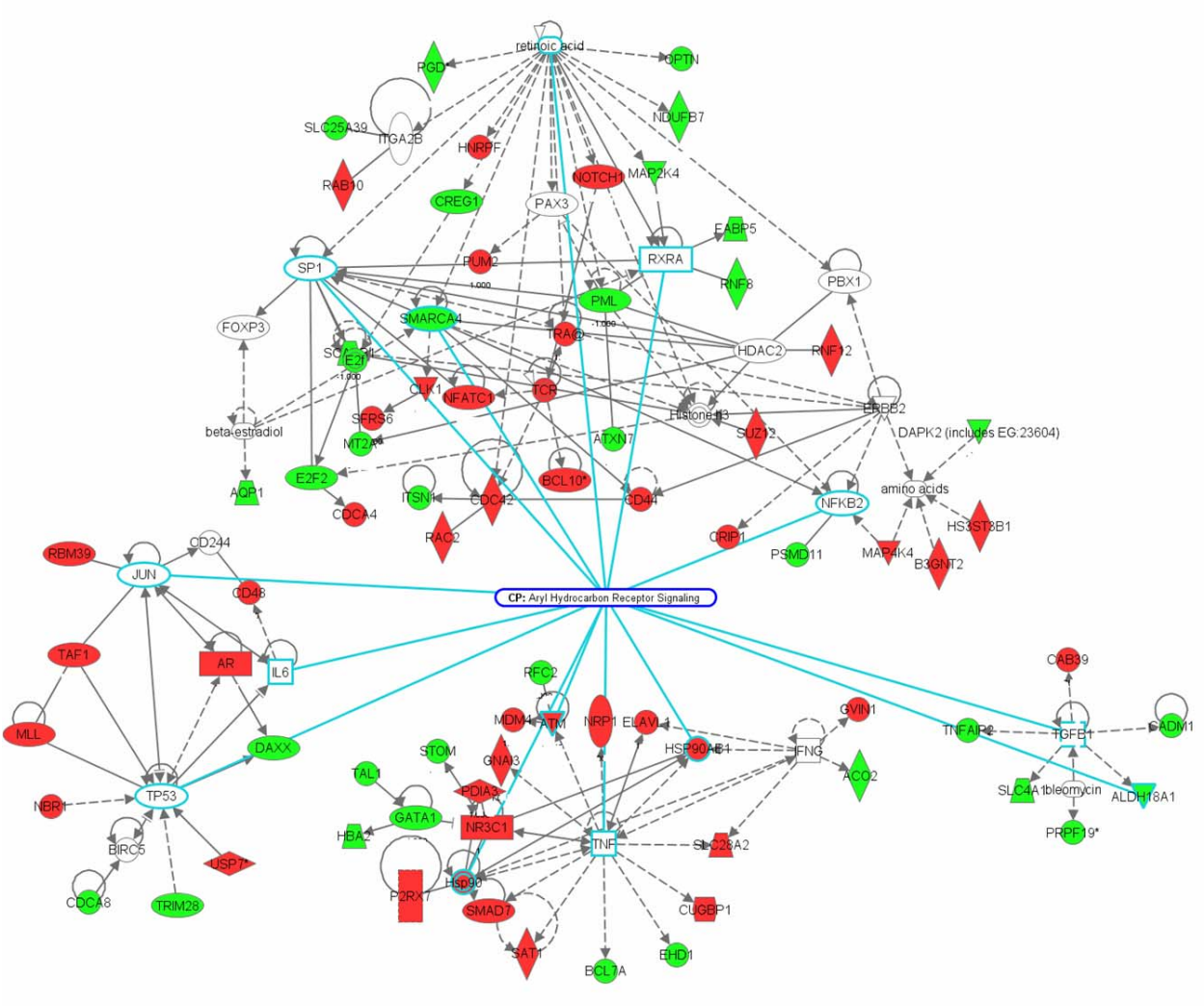

Figure 5-7. Pathway analysis of differentially expressed genes in $CD4^+$ T cells isolated from B6 and B6.*Sle1a.1* mice. The red designation indicates genes with increased expression while the green designation indicates genes with decreased expression compared to normal B6. Our collaborator, Dr. Igor Dozmorov, analyzed microarray data from the B6.*Sle1a.1* $CD4^+$ T cells using pathway analysis software. Based on these clusters, a connection between *Pbx1* and RA was revealed.

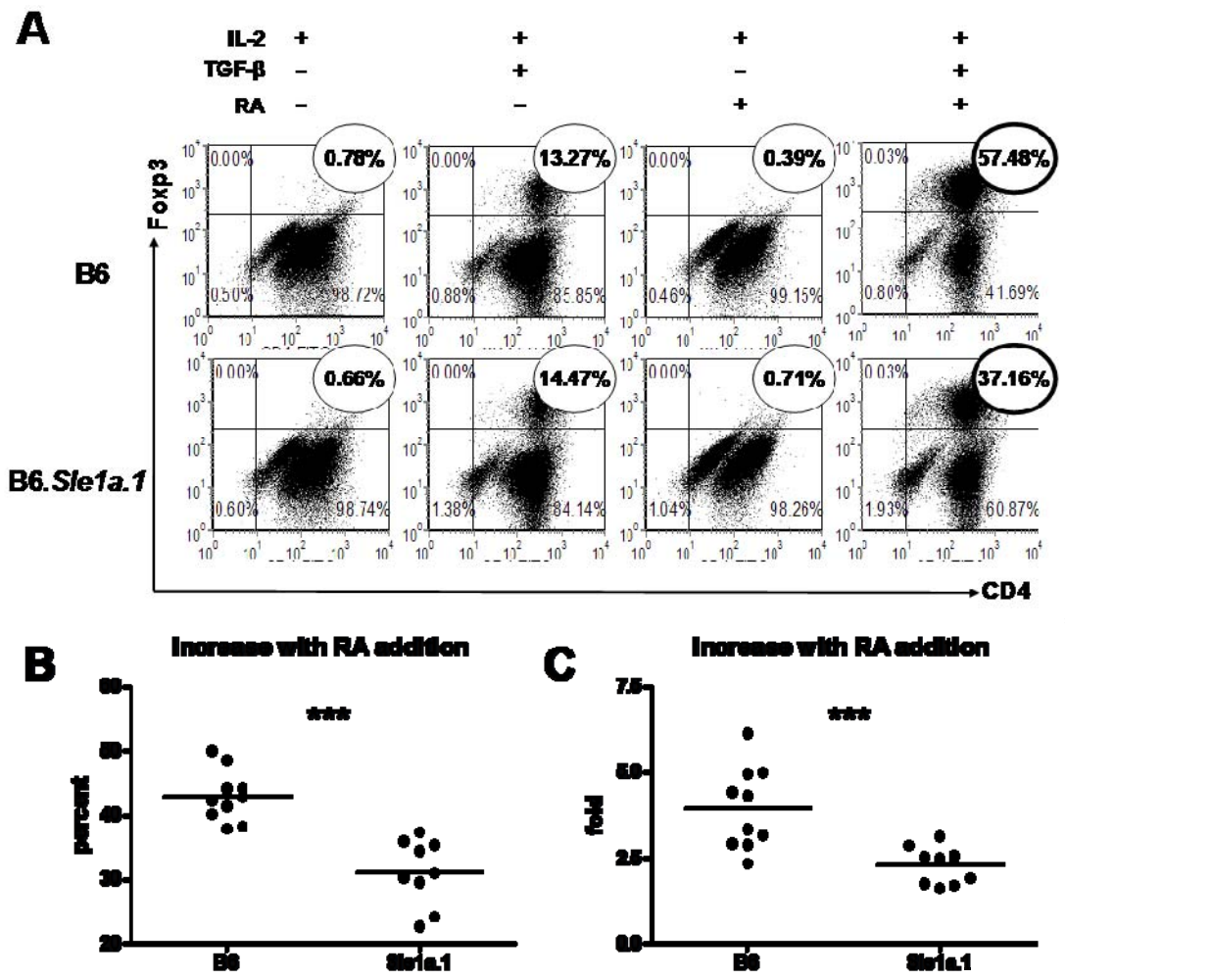

Figure 5-8. The NZW allele of *Pbx1* leads to a defect in RA-enhanced TGF-β-induced expansion of aTregs *in vitro*. CD4+ CD25- cells (5 x 10^5 cells/ml) from both 3 month old female B6 and B6.*Sle1a.1* mice were cultured with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (10 ug/ml), IL-2 (100 U) in the presence or absence of TGF-β (20 ng/ml) ± RA (5 nM) for 5 days and labeled for surface CD4 and intracellular Foxp3 expression and analyzed by fluorescence-activated cell sorting. A) Representative FACS plot depicting intracellular staining of Foxp3 expression by $CD4^+$ T cells. Quantification of $CD4^+$ $Foxp3^+$ cells measured as the percent (B) increase with RA addition and fold (C) increase with RA addition from the base TGF-β-induced population from 3 experiments. Two-tailed *t* tests: ***: $p<0.001$.

CHAPTER 6
DISCUSSION

We have shown that expression of *Sle1a* is sufficient to induce increased activation levels of $CD4^+$ T cells, DCs and B cells, as well as to down-regulate Treg levels. We also showed that *Sle1a* $CD4^+$ T cells express increased levels of the co-stimulation marker ICOS, which has been shown to play a critical role in T cell help to B cells, especially in germinal centers (170,171). Elevated ICOS expression on T cells from lupus patients has now been reported in three independent studies (172-174). These last two studies reported that ICOS stimulation of lupus T cells significantly enhanced anti-dsDNA Ab production from autologous B cells, which is equivalent to what we have shown for *Sle1a* T cells, which were able to induce anti-chromatin production in both autologous *Sle1a*-expressing B cells and normal B cells (13). These results also suggest that *Sle1a* confers a T cell phenotype that is found in lupus patients, which further validates the need to discover the identity of the *Sle1a* gene(s). Future experiments should address the specific role of ICOS in this process. High levels of ICOS have been associated with IL-10 secretion by $CD4^+$ T cells (175), and IL-10 production by $CD4^+$ T cells is significantly increased in the NZM2410 model (176). There was however no consistent increase of *ex vivo* IL-10 production by *Sle1a* $CD4^+$ T cells, suggesting that another mechanism may be involved. We assessed levels of ICOS expression in the subloci associated with *Sle1a* and found that while expression of *Sle1a.1* led to a significant increase in ICOS expression, this was not observed for expression of *Sle1a.2*. While *Sle1a* expression has also been shown to lead to a significant increase in CD69 expression on $CD4^+$ T cells, we did not observe this for either of the subloci, indicating that neither *Sle1a.1* nor *Sle1a.2* can fully account for the activation phenotypes associated with *Sle1a* expression.

Sle1a induces a reduction in the size of the Treg compartment, but these cells express normal levels of CTLA-4, CD103, and GITR, molecules which have been commonly associated with the regulatory phenotype (193-196). When we analyzed B6.*Sle1a.1* and B6.*Sle1a.2* strains for their Treg populations, we found that although there was a trend towards a decrease in the $CD4^+$ $CD25^+$ $CD62L^+$ population, it was not to the level of significance when compared with B6 values. Upon analysis of intracellular expression of Foxp3 among the $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^+$ $CD62L^+$ population, we found that only *Sle1a* and *Sle1a.2* populations expressed significantly decreased levels of this transcription factor known to commit cells to the Treg lineage. Again, these results indicate that neither sublocus can completely account for the decrease in the Treg compartment associated with *Sle1a*. In addition, at the higher ratios of Treg:Teff, *Sle1a*-expressing Tregs are fully capable of suppressing the proliferation of B6 Teffs on a per-cell basis in the presence of B6 APCs. However, at lower ratios of Treg:Teff, this suppressive capability is decreased, consistent with a reduced proportion of functional Tregs within the $CD4^+$ $CD25^+$ T cell population of the B6.*Sle1a* mice. However, we saw that at all ratios of Treg:Teff, both *Sle1a.1* and *Sle1a.2*-expressing $CD4^+$ $CD25^+$ Tregs were capable of suppression equal to that of normal B6 $CD4^+$ $CD25^+$ Tregs. In addition to *in vitro* suppression assays, we also performed adoptive transfers adapted from the experimental model of colitis to test the *in vivo* effect of *Sle1a* on Treg and Teff functions in a rapid model of disease. Results from the *in vivo* study confirmed our *in vitro* data for *Sle1a*, while we saw mixed results for the subloci. The *Sle1a.2*-expressing Tregs were less able to prevent colitis onset than normal B6 Tregs, but were not as defective in function as the *Sle1a*-expressing Tregs. We cannot exclude, however that *Sle1a* also affects Treg inhibitory functions. Indeed, a recent construct with a non-functional Foxp3 has demonstrated that the expression of Treg signature makers can develop

normally in cells that completely lack inhibitory functions (177). A definitive answer to that question will require the full panel of experiments involving *Sle1a*-expressing mice, along with *Sle1a.1* and *Sle1a.2*-expressing mice, bred to a Foxp3 reporter construct, which we are currently pursuing. We have found that the B6.*Sle1a.1*.FOXP3-eGFP mice at a young age present with a significantly decreased ratio of $CD4^+$ $Foxp3^+$ T cells to total $CD4^+$ T cells, but analysis of this cell population's function is required and is currently being pursued.

While we have shown that the *Sle1a*-expressing Tregs are capable of suppression, in situations where either the Teffs or the APCs express *Sle1a*, the suppressive capability of normal B6 Tregs is significantly reduced, suggesting that the *Sle1a* locus confers a resistance to suppression of Teff proliferation and that the APCs are playing a role in this phenomenon (189). We performed these same experiments with cells expressing *Sle1a.1* and *Sle1a.2*, and found similar results. It would appear that expression of *Sle1a.1* and *Sle1a.2* in Teffs or APCs confers resistance to suppression by normal Tregs, although not to the extent observed for *Sle1a*-expressing Teffs or APCs. This is yet further evidence validating our hypothesis that expression of both *Sle1a.1* and *Sle1a.2* are necessary to observe the full effect of *Sle1a* expression. It is of note that the APC population used in our *in vitro* suppression assays contains not only DCs but B cells as well. We have previously shown the effects of *Sle1a* DCs on Treg suppression (19), however, *Sle1a* affects both of these cell types. This indicates a potential role of activated B cells on Treg function, and is an avenue to be studied further. A similar Teff resistance has been previously reported in another model of lupus (162), but it is not clear at this point whether this resistance is the mere consequence of hyperactivation, or a result of involvement with a specific mechanism. *Cbl-b* deficiency results in a resistance to Treg regulation involving TGF-β, and this mutation also induces an increased level of activation in effector T cells (178). To our

knowledge, no other mechanisms of resistance to Tregs have been reported and additional experiments will be necessary to determine how *Sle1a* confers this resistance in $CD4^+$ T cells. We have previously shown that DCs from the NZM2410 triple congenic strain, B6.TC, prevent Tregs from performing their inhibitory functions, primarily through the production of IL-6 (158). We report here that *Sle1a*-expressing DCs present the same phenotype of high IL-6 production and Treg inhibition, indicating that this locus plays a major role in the overall DC phenotype of lupus-prone mice. Interestingly, the type-1 diabetes prone NOD mice, which have a reduced number of Tregs (197,180), also produce APCs that fail to fully support Treg functions (180). These results suggest that defective support or active inhibition of Treg functions by DCs may be a common mechanism affecting autoimmune pathogenesis.

Mixed BM chimeras have shown here that the increased proliferation and activation of *Sle1a*-expressing T cells, as well as the reduced *Sle1a* Treg level require that *Sle1a* be expressed in these T cells. Similar results were obtained for both *Sle1a.1* and *Sle1a.2*. These results differ from what might have been predicted from the *in vitro* reconstitution experiments shown in Figure 3-5, where B6.*Sle1a*-derived APCs inhibited Treg function. The BM chimera results do not mean that *Sle1a* exclusively affects $CD4^+$ T cells. In an analogous set of experiments, BM chimeras showed that T cell activation and autoreactivity mediated by *Sle3* were the indirect result of *Sle3* expression within the myeloid compartment (62,181). It is therefore possible that the *Sle1a*-induced intrinsic defects in $CD4^+$ T cells are indirectly responsible for the DC and B cell abnormalities. Alternatively, the *Sle1a* gene(s) may control a pathway present in all three cellular compartments. In any event, indirect or direct activation of DCs by *Sle1a* was not sufficient to convey extrinsic changes to B6-derived $CD4^+$ T cells *in vivo.* The exact cause for these differences is unclear, and highlights the need to confirm *in vitro* findings with *in vivo*

studies. Additional mixed BM chimeras will be necessary to address whether *Sle1a* expression in these DCs and B cells is necessary for production of the activated phenotypes.

Autoreactive T cells are a feature common to many autoimmune diseases for which a genetic basis has been demonstrated, yet only very few genes have been identified as responsible for this phenotype (182). In addition to *Cbl-b* discussed above (178), null alleles of *Gadd45a* (183) or *E2f2* (184) result in a lower threshold for T cell activation culminating in autoimmune pathogenesis, while null alleles in *Ctla4* (185) and *Foxp3* (198) result in massive inflammatory and autoimmune responses through the disruption of the Treg compartment. More recently, a natural polymorphism in the *Il2* gene has been identified as responsible for the diabetes susceptibility locus *Idd3* in the NOD mouse, also through an impairment of Treg function (199). The *Sle1a* interval does not contain any gene with obvious function in T cells. Our *in vitro* results showed that *Sle1a* confers an autoimmune phenotype to $CD4^+$ T cells in the colon, which is not typically associated with lupus pathogenesis. This indicates that *Sle1a* affects a genetic pathway regulating production of Tregs and responses to Tregs in a manner that is not restricted to tolerance to nuclear antigens. The identification of the *Sle1a* gene(s) will therefore uncover a novel and broad pathway by which autoreactive T cells are regulated by Tregs.

Using Ensembl Realease 40, we found that only one gene exists in the *Sle1a.1* region of chromosome 1, *Pbx1*, which encodes for a transcription factor necessary for transcription of multiple genes. There is 100% amino acid sequence homology between mouse and human Pbx1, and it is therefore a possibility that we may find similar results among SLE patients as we found in our SLE mouse model. We intend to pursue this further by analyzing a large cohort of SLE patients with age- and sex-matched controls. It would also be of use for us to group patients with

active SLE versus inactive SLE and correlate the number of Tregs in their peripheral blood to the isoforms of Pbx1 present in their T cells.

TGF-β has been shown to induce Foxp3 expression. Activin, a member of the TGF-β family of growth factors, is known to be a critical regulator of follicle-stimulating hormone (FSH) expression by activating transcription of the FSH-β subunit and stimulating FSH secretion (200-202). Like all members of the TGF-β family, activin signals through serine/threonine kinases, which then phosphorylate intracellular receptor-specific Smad proteins, Smad2 and Smad3 in the case of activin (203). Smad6 or Smad7 has the capacity to block this phosphorylation, thereby blocking translocation to the nucleus (204). In 2004, it was published that three activin-responsive regions are required for full activin response with smad-binding elements (SBE) present in all (205). One of these elements was found to bind a complex containing Pbx1, Prep1 and Smad4, identifying Pbx1 and Prep1 as Smad binding partners and mediators of activin action (205). It has been shown that both Meis and Prep associate with Pbx1 in the cytoplasm and induce a conformational change in Pbx1, exposing the nuclear localization signal, and subsequently causing translocation of the dimeric protein complex to the nucleus (144). Treatment with RA has been shown to expand both Meis and Pbx1 expression in various cell types (114,146,147). Since RA was shown to potently synergize with TGF-β in driving Foxp3 induction (127), it is of interest to elucidate this mechanism. RA has been implicated in enhancing TGF-β signaling by increasing the expression and phosphorylation of Smad3, resulting in increased Foxp3 expression (133).

In our case, we propose that RA-enhanced TGF-β-induced expression of Foxp3, and therefore the production of aTregs, is mediated by Pbx1. In a normal situation (Fig. 6-1A), TGF-β binds the serine/threonine kinases, resulting in the phosphorylation of intracellular receptor-

specific Smad3, which then binds with Smad4, and this complex can then translocate to the nucleus. Normal amounts of Smad7 would not block this process. Smad3/4 complexes can bind DNA alone, but with low affinity, so their interaction with additional transcription factors is required for target gene regulation. Since the transcription factor Pbx1 has been shown to bind with Smad partners, we postulate that the partner is Smad3, and that this interaction leads to enhanced binding to a target gene, perhaps Foxp3 in this case. RA has been involved in increasing Pbx1 expression via transcriptional regulation as well stabilization of Pbx1 proteins, and shown to be likely related to an increase in the association between Pbx1 and Meis. The binding of Meis induces a conformational change in Pbx1, exposing the nuclear localization signal, and subsequently causing translocation of the dimeric protein complex to the nucleus, where it can bind DNA but with low affinity as well. A complex involving Meis/Pbx1 and Smad3/4 may be the connection between the RA and TGF-β signaling pathways.

In our *Sle1a.1*-expressing T cells (Fig. 6-1B), where the NZW allele of *Pbx1* is expressed, we have a shown that there is a reduced induction of Foxp3 expression with RA addition to the culture with TGF-β. Since the NZW allele of *Pbx1* leads to expression of an alternative isoform predicted to lack the DNA binding domain, we hypothesize that Pbx1-c, present in both *Sle1a.1* and normal T cells can carry out the normal function of binding the Smad3/4 complex to transcribe target genes, but Pbx1-d, found only in the *Sle1a.1*-expressing T cells, is unable to do so. Also, as shown in Table 5-1, the *Sle1a.1* T cells express significantly more Smad7, a factor known to block the phosphorylation of Smad3, thereby rendering it incapable of binding Smad4 and subsequently translocating to the nucleus. Therefore, there is an overall reduction in transcription of target genes in the *Sle1a.1*-expressing T cells, leading to a decreased production

of aTregs. It should be noted that there is the potential role for RA in differential splicing of target genes as well, in this case *Pbx1*.

Further studies involving molecular protocols are in preparation to test this hypothesis. Since we have found that Pbx1-d seems to be expressed at higher levels in patients with SLE, we have begun studies involving a human T cell line, the Jurkat T cell line, in order to test the effect of overexpression of the Pbx1-d isoform on T cell function. Our collaborator has made a viral construct of Pbx1-d and infected Jurkat T cells, which do not normally contain this truncated isoform. We are currently in the process of performing the aforementioned assay involving RA-enhanced TGF-β-induced production of aTregs using the virally-infected Jurkat T cells compared to those Jurkat T cells infected with a random gene. Should we observe that there is a decrease in the production of aTregs, we can definitively say that Pbx1 is involved in mediating RA-enhanced production of aTregs and is an integral transcription factor in this mechanism. We could also potentially test the levels of phosphorylated Smad3 between normal B6 and *Sle1a.1*-expressing T cells, or possibly test the level of binding activity of Smad3 to Pbx1 to see if we can definitively prove that Pbx1 is the factor that links the RA- and TGF-β-signaling pathways.

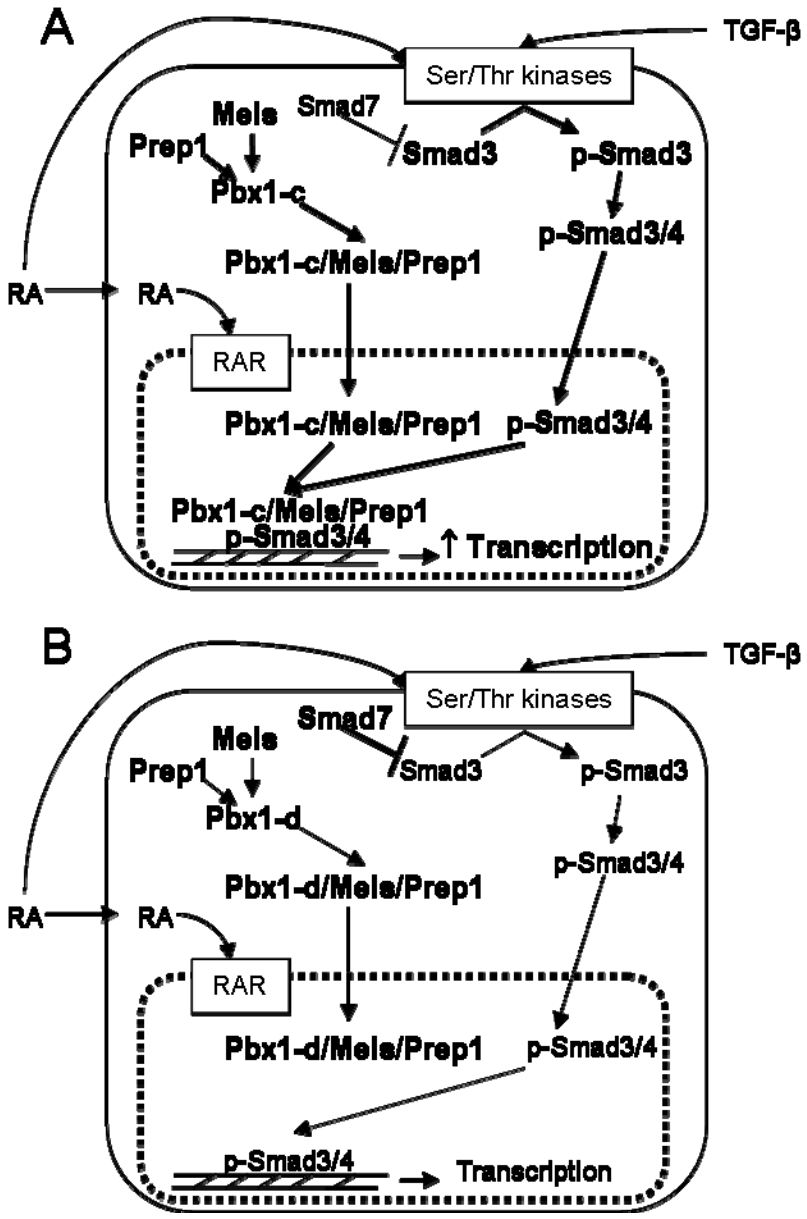

Figure 6-1. The proposed mechanism of Pbx1's role in the connection of the retinoic acid and TGF-β signaling pathways in a T cell. Retinoic acid can enhance TGF-β-induced signaling with the binding of the Pbx1-c complex (A) to the Smad complex in the nucleus leading to increased transcription of target genes when bound to DNA, but since Pbx1-d lacks the DNA binding domain, less transcription occurs when this isoform is present (B).

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BIOGRAPHICAL SKETCH

Having lived all over the country as a young girl, Carla chose to attend the University of Florida after graduating in May 1999 with honors from Gainesville's own Eastside High School International Baccalaureate (IB) program. An advanced placement high school biology class led her to choose microbiology and cell ccience as her major with a minor in chemistry. By her senior year, she was set on pursuing a Ph.D. in biomedical research and applied to schools around the nation offering interdisciplinary programs. She graduated cum laude in the Spring of 2003 with her Bachelor's and remained at the University of Florida to begin her Ph.D. studies in the Fall of 2003. The College of Medicine's Interdisciplinary Program in Biomedical Research gave her many options as to which discipline to choose. An interesting undergraduate class in immunology as well as a core course in the interdisciplinary program in immunology and microbiology led her to choose a research path in that direction. In the Spring of 2004, after a six week rotation, Dr. Laurence Morel offered her a position as a Graduate Research Assistant in the Department of Pathology, Immunology and Laboratory Medicine studying a congenic murine model of systemic lupus erythematosus.