IN VIVO IDENTIFICATION OF *SLE1B*:

LY108 MEDIATES AUTOANTIBODY

PRODUCTION

APPROVED BY SUPERVISORY COMMITTEE

Edward Wakeland, Ph.D.

David Karp, M.D., Ph.D.

Anne Satterthwaite, Ph.D.

Nicolai Van Oers, Ph.D.

Pamela Schwartzberg, M.D., Ph.D.

ACKNOWLEDGEMENTS

I want to first thank my mentor, Ward Wakeland, who provided me with an enlightening lab experience and always believed in my abilities as a scientist. He gave me challenging projects that pushed me and helped me to realize my passion for science.

I also want to thank my dissertation committee members for their time and patience with me.

No words can even describe the amount of support I have received from two very important people in my life, Michael and Charles. They have always provided a listening ear to all my troubles even in their sleep-deprived, post-call state from the hospital. And I cannot even count the number of times they have even donned a pair of gloves to help me in lab when I have gotten myself way over my head in work.

Two members of my lab, Ferdicia and Nana, who have always been there to provide me with the lessons they have learned from years of lab and life experiences. They have helped me troubleshoot so many lab problems both technically and personally.

I am also indebted to Vidya, who without her constant support and encouragement, this dissertation would not have existed.

Thank you to my MSTP friends and graduate school colleagues both inside and outside of

lab for their scientific and technical discussions and most importantly their friendship.

Lastly, I need to thank my parents and family who I am sure had serious doubts whether I would ever finish my time in lab, and are relieved to see me finally moving forward again. Thank you for always supporting me.

I hope I can fulfill all the hopes and aspirations you all have in me. I will always be indebt to all of you for all of your constant encouragement and support.

IN VIVO IDENTIFICATION OF *SLE1B*: LY108 MEDIATES AUTOANTIBODY PRODUCTION

by

ALICE YANAN CHAN

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

June, 2006

IN VIVO IDENTIFICATION OF *SLE1B*:

LY108 MEDIATES AUTOANTIBODY

PRODUCTION

Publication No. _____

Alice Yanan Chan, Ph.D

The University of Texas Southwestern Medical Center at Dallas, 2006

Supervising Professor: Edward K. Wakeland, Ph.D

In the NZM2410 model of murine lupus, *Sle1b* mediates anti-nuclear autoantibody (ANA) production. Our goal is to determine the causative gene in the *Sle1b* locus. Seven members of the SLAM/CD2 family are located within the *Sle1b* interval, and previous work has shown that structural and expression polymorphisms in lymphocytes distinguish two major SLAM/CD2 haplotypes. To further narrow the interval, we utilized a BAC transgenic rescue approach whereby BACs carrying the lupus-resistant B6 alleles were bred to B6.*Sle1b* mice to identify the region mediating ANA suppression. One BAC carrying *Cd84* and *Ly108* suppressed autoantibody production. We then generated BAC transgenic mice carrying the lupus-susceptible (129) and lupus-resistant (B6) alleles of *Ly108* on the B6 and B6.*Sle1b*

genetic background, respectively. The B6 allele of *Ly108* suppresses ANA production on the lupus-susceptible B6.*Sle1b* background while the 129 allele induces ANA on the lupus-resistant B6 genome. Taken together, these data identify *Ly108* as a causative gene in *Sle1b*. While *Ly108* is needed to mediate the breach in tolerance, we have also identified other SLAM family members as genetic modifiers necessary to recapitulate fully penetrant, high titer ANA production as seen in *Sle1b*.

We found that *in vitro* stimulation of B6.*Sle1b* CD4 T cells led to altered cytokine production, such as decreased IL4 production. Interestingly, these phenotypes have also been reported in knockouts of SLAM/CD2 family members as well as in the absence of the SLAM family adaptor, SAP. Our data indicates that the presence of the *Sle1b* haplotype, derived from either NZM2410 or 129, recapitulates these phenotypes, independent of the absence of these molecules. While recent reports have suggested a role for SAP in ANA development, we find that the breach in tolerance in *Sle1b* mice is SAP-independent. However, SAP is necessary to potentiate the autoantibody production.

ANAs is an important biomarker for autoimmune diseases including, Systemic Lupus Erythematosus (SLE), and potentially identifies an autoimmune-prone state. We have identified genes which contribute to the production of ANAs. Elucidating the pathways these genes dysregulate will provide critical insight into our understanding of tolerance and how tolerance can be breached.

.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Wandstrat, AE, Nguyen C, Limaye N, **Chan AY**, Subramanian S, Tian XH, Yim YS, Persemlidis A, Garner, Jr. HR, Morel L, and Wakeland EK. (2004) Association of extensive polymorphisms in the SLAM/CD2 gene cluster with murine lupus. *Immunity* 21(6):769-780.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

129 – 129Sv/J

ANA – Anti-nuclear Autoantibodies

B6 – C57Bl/6

BAC – Bacterial Artificial Chromosome

dsDNA – double stranded DNA

EAE – Experimental Autoimmune Encephalomyelitis

EAT-2 – Ewing's sarcoma-associated transcript-2

EBV – Epstein Barr Virus

ERT – Eat-2-related transducer

GN – Glomerulonephritis

GPI - Glycophosphatidylinositol

Ig – Immunoglobulin

ITSM – Immuno-Tyrosine based Switch Motif

Kb – kilobases

MCTD – Mixed Connective Tissue Disease

MS – Multiple Sclerosis

NOD – Nonobese Diabetic Mouse Model

NZB – New Zealand Black

NZM – NZM2410

NZW – New Zealand White

RA – Rheumatoid Arthritis

RF – Rheumatoid Factor

SAP – Signaling Lymphocyte Activation Molecule (SLAM)-Associated Protein

SLE – Systemic Lupus Erythematosus

SNP – Single Nucleotide Polymorphisms

T_{FH} – follicular T helper cell

XLP – X-linked Lymphoproliferative Disease

CHAPTER ONE

Introduction

AUTOIMMUNITY

Autoimmunity arises when immune regulatory mechanisms fail to maintain tolerance to self antigens. This breach in tolerance leads to the generation of pathogenic autoantibodies and autoreactive cells that attack various organ systems culminating in a spectrum of clinical manifestations. Collectively, autoimmune diseases are reported to affect 5-7% of the Western population (1, 2). They can be classified into two main groups according to the scope and nature of the tissue damage: organ-specific or systemic. Organ-specific diseases include type I diabetes mellitus or Hashimoto's thyroiditis, which target the pancreas and thyroid, respectively. In contrast, in systemic autoimmune diseases pathological damage occurs in multiple different organs and tissues, and the prototypical example of this type of disorder is systemic lupus erythematosus (SLE).

AUTOIMMUNE DISEASES WITH SPECIFIC ANTI-NUCLEAR AUTOANTIBODIES

Systemic autoimmune diseases are commonly associated with the development of autoantibodies recognizing cytoplasmic and nuclear components. The presence of specific subsets of serum autoantibodies is commonly used as the initial criteria for disease diagnosis. The importance of these autoreactive antibodies to the development of organ and tissue destruction remains inconclusive, and it is possible that their presence may simply reflect an

immune state that is gradually becoming more autoreactive. It has, however, been established that development of autoantibodies precedes the onset of clinical symptoms, in some cases by many years. In a recent retrospective study conducted by Arbuckle and colleagues on serum repositories from members of the military, 88% of patients showed at least one SLE autoantibody prior to the development of SLE, and 78% were positive for anti-nuclear autoantibodies (ANAs). Moreover, ANAs and other antibodies were detected as early as three years prior to disease diagnosis thus serving as a potential biomarker for SLE predisposition (3). Although ANAs have been classically associated with SLE and other rheumatic disorders, it can also be found in patients with lymphoproliferative diseases, as well as in certain cases of chronic infections. The presence of low titers of ANAs can even be found amongst healthy adults: 32% at 1:40 dilution and 5% at a 1:160 dilution (4). This population may actually represent a pool of individuals that are genetically predisposed to the development of autoimmune diseases, and similar to the ANA-positive military personnel, some may ultimately progress towards disease. There are four systemic diseases that are classically associated with the development of autoantibodies towards specific anti-nuclear antigens: systemic sclerosis, mixed connective tissue disease, Sjogren's syndrome, and SLE.

Systemic Sclerosis

Systemic sclerosis, also known as scleroderma, is a chronic disease affecting multiple organ systems that is characterized by the progressive accumulation of connective tissue that ultimately leads to thickening of the skin and dysfunctions of various visceral organs. The disease begins around 30 to 50 years of age. The patients usually develop a predominantly

polyclonal IgG hypergammaglobulinemia, and 95% of patients are ANA positive. These ANAs are commonly found to be directed against DNA topoisomerase I, Scl-70, and other centromeric proteins (4). There are various induced and genetic models of systemic sclerosis, including the bleomycin-induced and tight-skin-1 and -2 models (5).

Mixed Connective Tissue Disease

The disorder known as Mixed Connective Tissue Disease (MCTD) actually represents a compilation of symptoms observed in SLE, polymyositis, and rheumatoid arthritis (RA). Patients typically present with joint and muscle pain, and are usually women in their 20 to 30s. The distinguishing feature of this disease is the presence of very high titer ANA against ribonucleoprotein, U1RNP (4). Immunizing C57Bl/6 (B6) mice with the U1RNP antigen leads to the development of a phenotype that closely resembles MCTD (4).

Sjögren's Syndrome

Sjögren's syndrome results from lymphocytic infiltration of various exocrine glands and leads to symptoms like dry mouth and dry eyes. These patients can also develop gastrointestinal and respiratory tract problems resulting from decreased mucus production in these systems. Similar to most autoimmune diseases, patients with Sjogren's syndrome are typically female, and they commonly develop autoantibodies to the ribonucleoproteins, Ro/SS-A and La/SS-B. Patients with other autoimmune diseases have also been known to develop secondary Sjögren's syndrome (4). The MRL/*lpr* and nonobese diabetic (NOD) mouse strains are models for studying this disease as well as lupus and type I diabetes,

respectively (6, 7).

Systemic Lupus Erythematosus (SLE)

SLE, the prototypical systemic autoimmune disease, primarily affects women and commonly begins in their child-bearing age. It has an incidence of approximately 1 in 2000 individuals, and some studies have reported frequencies as high as 1 in 800 (4, 8). Majority of patients have elevated titers of ANAs, and antibodies specific for double-stranded DNA (dsDNA) and the Sm protein are key diagnostic markers for SLE. The disease is characterized by a chronic abnormal immune response that potentially targets all major organ systems. This leads to a heterogeneous clinical presentation that ranges from mild symptoms like fatigue, arthritis, and the characteristic malar rash, to more severe conditions like renal and neurologic destruction. The disease course will oftentimes wax and wane but will progressively lead to an overall impairment of organ function that is associated with significant morbidity and mortality.

The underlying etiology of this complex disease remains largely unknown, but a significant body of research has demonstrated that both environmental and genetic factors impact its development and severity. It has been shown that genetic predisposition is the strongest factor influencing disease development. Classic studies in twins have found concordance rates approximately ten-fold higher in monozygotic (34%) than in dizygotic (3%) twins (9, 10). It has been well established that SLE, similar to many other autoimmune diseases, has a complex polygenic inheritance pattern such that multiple susceptibility loci are interacting

with each other to mediate disease pathogenesis (11). Due to the complex genetic nature of the disease, it has been difficult to conclusively identify potent susceptibility loci in humans. A variety of spontaneous mouse models of lupus have been used as an alternative to assist in the identification of causative genes and the underlying molecular mechanisms behind this complex disease.

COMMON SUSCEPTIBILITY GENES FOR AUTOIMMUNE DISEASES

Diagnosis of autoimmune disorders requires fulfilling set criteria for the specific disease; however, many of these diseases share overlapping clinical features. Up to 25% of patients diagnosed with one rheumatic disease will also have symptoms for other autoimmune diseases and are thus classified as having overlap syndrome (12). Furthermore, it is known that multiple autoimmune diseases can run in a given family, which suggests that there may be susceptibility genes shared among the various disorders (13-16). This is supported by the recent evidence of an allele for *PTPN22*, a phosphatase involved in regulation of T cell activation, that is associated with type I diabetes, RA, SLE, and Hashimoto's thyroiditis (17). Thus, identifying causative alleles in one autoimmune disease may also shed light on other autoimmune diseases. Our lab focuses on the NZM2410 murine model of SLE to not only identify genes involved in lupus susceptibility but also discover underlying pathways that are common to the 'autoimmune state'.

MURINE MODEL OF SLE: NZM2410 AND ITS SUSCEPTIBILITY LOCI

The NZM2410 (NZM) strain is one of the classic spontaneous models of murine lupus. This

recombinant inbred strain was generated from an [NZB X NZW]F_1 X NZW backcross, such that 75% of its genome is derived from NZW and 25% from NZB (18, 19). Both males and females from this strain develop a highly penetrant form of murine lupus, with females having a slightly earlier age of onset (20). As these mice age, ~80% of them will develop anti-dsDNA autoantibodies, and progress towards the development of fatal glomerulonephritis (GN) (20). Both of these characteristics are highly reminiscent of the clinical presentation seen in SLE patients.

To identify genetic loci mediating lupus susceptibility in this model, linkage analysis on progeny from an [NZM X B6]F_1 and NZM backcross revealed the presence of multiple NZM loci mediating the autoimmune phenotype. This led to the discovery of *Sle1* on chromosome 1, *Sle2* on chromosome 4, *Sle3* on chromosome 7, and the H2 region on chromosome 17. The first three loci were shown to act in recessive manner, whereas the latter locus enhanced GN susceptibility in heterozygotes (20).

To determine the phenotypes mediated by each of the individual loci, a congenic dissection approach was used, whereby each susceptibility loci was introgressed onto the B6 genome using microsatellite marker assisted screening (21). This provided a system to characterize each locus independently to identify the individual pathway that each locus was involved in. These analyses essentially converted a complex, polygenic trait into a series of monogenic phenotypes. Characterization of the immunological and pathological phenotypes mediated by these isolated loci and when these various loci were recombined, led to our current proposed

model for disease pathogenesis (Figure 1). This model postulates that the key initiation step is the loss in tolerance to chromatin mediated by the *Sle1* locus in our model. The generation of these ANAs can be possibly attributable to modulations in immune cell interactions and responses, as well as changes in antigen clearance. The second step involves genes that potentiate changes in the overall immune responsiveness, leading to further dysregulation of immune function and properties. Finally, susceptibility genes belonging to the final stage are believed to exacerbate end-organ damage by modifying inflammatory responses specifically to the target organs. We have identified susceptibility loci belonging to each of these stages. By reassembling all the individual loci onto the lupus-resistant B6 genome, the disease phenotypes and penetrance were fully recapitulated, suggesting that all the major genes involved in lupus susceptibility in the NZM2410 model were identified. It was further shown that increasing the number of susceptibility alleles led to increasing disease probability, mirroring the threshold liability model (22).

Sle1 **Locus**

Based on the studies described above involving various combinations of the different loci, *Sle1* was identified as the necessary locus for the initiation of pathogenic autoimmunity in our model system (22). *Sle1* leads to a breach in tolerance to nuclear antigens specific for H2A/H2B/dsDNA subnucleosomes (23). By 9 to 12 months of age, 75% of the mice develop high titers of anti-chromatin IgG autoantibodies, with a predilection towards an earlier onset in females (23). B6.*Sle1* mice also develop splenomegaly and have a progressive increase in the expression of activation markers on CD4 T cells and B cells (23, 24). Despite having an

intrinsic activation phenotype, these cells fail to show a difference in their proliferative capacity to various B and T cell stimuli or a difference in apoptosis (23). However, based on mixed bone marrow (BM) chimera studies, the B cell compartment from *Sle1* has been shown to be necessary for mediating the production of anti-chromatin autoantibodies. Furthermore, using mice lacking a T cell receptor (TCR), it has been shown that autoantibody production and the increased B cell activation marker phenotypes were independent of T cells. Similarly, the increased T cell activation marker phenotypes and cytokine production was intrinsic to the *Sle1*-expressing T cells in the absence of B cells (24, 25).

Sle1b **Locus**

Further fine-mapping analyses of the *Sle1* interval using congenic recombinants revealed that *Sle1* is composed of four sub-loci termed *Sle1a*, *Sle1b*, *Sle1c*, and *Sle1d* (26). Of all four loci, *Sle1b* was the strongest for mediating autoantibody production, with a penetrance of 93% in females and 72% in males (26). These antibodies had the same H2A/H2B/DNA subnucleosomal specificity as that seen in *Sle1*, and these mice also exhibited hypergammaglobulinemia (26). Upon OVA immunization, it was shown that B6.*Sle1b* mice had a higher IgM response in comparison to control mice (26). Also, this locus by itself mediates the progressive increase in activation markers on B and T cells, similar to that seen in *Sle1*. Consistent with these increased cell-surface activation phenotypes, CD4 T cells from *Sle1b* 2 month old mice had increased calcium flux responses to T cell stimuli *ex vivo*, indicative of an intrinsic hyper-responsiveness (27). These mice however did not show

differences in proliferation to either B or T cell stimuli as measured by CFSE dilution assays *in vitro* (28). By 9 to 12 months of age, B6.*Sle1b* mice showed an overall decrease in the percentage of CD8 T cells, with a predominance of activated CD4 cells and increased effector-memory CD4 cells that displayed a phenotype reminiscent of replicative senescence when stimulated *in vitro* (S. Subramanian, *unpublished results*). Overall, the phenotypic consequences of the *Sle1b* locus bear the most resemblance to *Sle1* phenotypes.

To identify the causative genes, a series of overlapping B6-derived Bacterial Artificial Chromosomes (BACs) spanning this segment was identified and sequenced to determine what genes were within the critical interval. The region had numerous single nucleotide polymorphisms (SNPs) distinguishing B6 and B6.*Sle1b* in both their structural and regulatory regions. The majority of SNPs were found within genes belonging to the SLAM family. Seven members of this family: 2B4, Ly9, Cs1, SLAM, CD48, CD84, and Ly108 are contained with this interval. The SLAM family member genes, *Ly9*, *Cs1*, *Cd48*, and *Cd84*, collectively had 12 non-synonymous SNPs that could potentially modify the functional properties of these molecules. Another member, *2b4*, expanded from one gene in B6 to four genes in *Sle1b*, with one mutated to a pseudogene due to the presence of an early stop codon (27-29).

Further analysis of the ligand-binding domain of these family members in both inbred and wild-derived strains delineated a haplotype block, a set of SNPs inherited as a single unit. The more prevalent haplotype found in the strains analyzed is the one shared with the

autoimmune-prone mouse, B6.*Sle1b*. Moving this haplotype from either Cast/Ei or 129 strains onto the B6 genomic background also led to the production of ANAs similar to B6.*Sle1b* mice. This suggests that the alleles mediating autoimmune susceptibility are actually common alleles, and that autoimmunity likely arises as a consequence of unfortunate combinations of common alleles in specific genomic contexts (27, 29).

In addition to these genomic analyses, RNA expression differences for all potential candidate genes were examined. Among the genes expressed in B and T cells, only those belonging to the SLAM family showed statistically significant differences between B6 and B6.*Sle1b*. Northern blot analysis on family members within the interval revealed differences in isoform usage in *Ly9* and *Ly108* between the two strains. However, only the *Ly108* isoform changes correlated with the autoimmune-promoting haplotype, strongly supporting its role as a causative allele (27, 28).

The autoimmune phenotype seen in B6.*Sle1b* is inherited in an allele-dose dependent manner, such that mice heterozygous for the *Sle1b* locus has a reduced penetrance of ANA (30%) when compared to homozygous mice (90%). This inheritance pattern enabled us to employ a BAC transgenic 'rescue' strategy. By introducing BACs carrying the B6 alleles of the *Sle1b* candidate genes as transgenes on the B6.*Sle1b* background, we can identify the causative gene for mediating the ANA phenotype. Only the BAC carrying the causative gene would lead to suppression and recapitulate the heterozygous state. Previous work by N. Limaye demonstrated that none of the non-*Slam* alleles were able to suppress ANA production. Two SLAM-family BACs, BAC25 (*2b4*, *Ly9*, and *Cs1*) and BAC40 (*Cd48* and

Slam), were also unable to suppress ANA production to a significant degree. However, BAC25 carrying *2b4*, *Ly9*, and *Cs1* showed a slight and consistent decrease in autoantibody production at 9 months but were not statistically significant (29). Among all the candidate genes, only one BAC harboring *Cd84* and *Ly108* genes remained to be determined.

SLAM FAMILY MEMBERS IN *SLE1B*

The *Sle1b* locus contains seven members of the SLAM family of genes: *2b4* (*Cd244*), *Ly9* (*Cd229*), *Cs1*, *Cd48*, *Slam* (*Cd150*), *Cd84*, and *Ly108*. Table 1 summarizes the genetic and expression differences present between B6 and B6.*Sle1b* mice in these family members. In general, these genes encode cell-surface receptors that participate in mediating either lymphocyte activation or inhibition, depending on both their isoform usage and cell-type expression. They contain at least two immunoglobulin (Ig)-like extracellular domains, a transmembrane domain, and a cytoplasmic tail containing immuno-tyrosine switch motifs (ITSM), TxYxxV/I, with the exception of CD48, which is a GPI-linked protein. These ITSMs can be phosphorylated by various molecules including members of the Src family of kinases. Phosphorylation leads to recruitment of downstream adaptors such as SLAM-associated protein (SAP, *Sh2d1a*), Eat-2 (Eat-2a, *Sh2d1b*), and ERT (Eat-2b, *Sh2d1c*). The following sections detail the ascribed functions of each family member in the *Sle1b* region. Table 2 summarizes features of the SLAM family members.

2B4 (CD244)

2B4 is expressed on a variety of immune cells, including subsets of γδ and CD8 T cells,

natural killer (NK) cells, as well as basophils and monocytes (30-32). Recently, it was also detected in hematopoietic progenitor cells (33). Unlike human cells where only one isoform is expressed, mice have two alternatively spliced isoforms that differ in their cytoplasmic tails and are called 2B4 long and 2B4 short. The long isoform contains four ITSMs whereas the short form has only one motif (34, 35). Following its interaction with CD48, the ITSMs in 2B4 can bind adaptors such as SAP, which can then recruit Fyn to phosphorylate molecules like Vav-1 to initiate signaling (36, 37). Characterization of the functional properties of 2B4 has primarily focused on NK cells and has demonstrated key roles in NK effector function, NK cell mediated self-tolerance, as well as NK-B cell interactions.

In vitro stimulation, using antibodies to 2B4, has shown an activating and inhibiting role in NK cytotoxicity depending on the isoform used; the long isoform functions as an inhibitory receptor while the short isoform behaves as a stimulatory receptor (38). Activation of NK cytotoxicity by the short form requires the SAP adaptor, whereas inhibition functions independent of SAP (39, 40). It has been speculated that the inhibitory function might be mediated by other adaptors for this family such as Eat-2 and ERT.

Using a knockout of the molecule generated in B6 ES cells, 2B4 appeared to play a predominant role as a negative regulator of NK cell function *in vivo*. The engagement of its ligand, CD48, was shown to inhibit NK-mediated lysis of tumor cells, as well as allogeneic and syngeneic targets (41). McNerney and colleagues further demonstrated that mice lacking both MHC class I and 2B4 have a more profound effect on tumor cell lysis and bone marrow

rejection than mice lacking only MHC class I (42). This suggests that the inhibitory function of 2B4 assists in maintaining NK self-tolerance, which had previously been thought to be mediated solely by self- MHC class I molecules. 2B4 has also been shown to play a role in NK-B cell interactions by modulating changes in cytokine production and B cell activation (43, 44). Taken cumulatively, it is possible that subtle alterations in any of these functions of this gene could potentially impact development of autoimmunity.

Ly9 (CD229)

Ly9 is expressed on thymocytes, mature T and B cells, and bone-marrow cells (45). Unlike other members of the family, this molecule contains four Ig-like domains that most likely arose from a duplication event (46). The extracellular domain contains eight potential N-linked glycosylation sites, and the cytoplasmic tail contains two ITSMs, similar to its human counterpart (47-49). Based on studies of mutant human Ly9, the first Ig domain is believed to mediate the homotypic interactions of this receptor (50). In mice there are two transcripts, 2.4 Kb and 3.2 Kb, as detected by Northern blot analyses (46). Based on sequence comparisons taken from the Ensembl database, the shorter transcript differs from the larger one in two aspects: (1) loss of the first IgV domain, which contains two potential N-glycosylation sites, and (2) use of an earlier start codon that introduces 21 additional amino acids at the N-terminus. Analysis of the promoter region revealed putative binding sites for several transcription factors such as GATA-2, NFAT-1, and PU.1 (51).

With the generation of a mouse deficient in Ly9, more functions of this molecule have been

uncovered. The mutant mice exhibited a defect in IL-4 production when T cells were stimulated, similar to the phenotypes seen in knockouts of SLAM and Ly108. The CD4 T cells also had suboptimal proliferation and reduced IL-2 secretion (52). The signals mediated through Ly9 are dependent on SAP binding, which then recruits Fyn and Lck to facilitate the full phosphorylation of the molecule (48, 53). Despite its expression in B cells, there is little known regarding the role of the Ly9 molecule in B cells. In addition, Ly9 expression has been shown to be elevated in follicular T helper (T_{FH}) cells, a subset of CD4 T cells that were recently implicated as being the major population dysregulated in the lupus-prone *san roquin* mouse (54, 55).

Cs1 (CD319, CRACC, 19A, novel Ly9, SLAMf7)

Cs1 is expressed on all NK cells, the majority of CD8 T cells, activated B cells and mature dendritic cells (56, 57). This cell-surface receptor, like most other members of the SLAM family, binds in a homotypic fashion via its two Ig-like extracellular domains (58). The mouse gene contains five putative N-linked glycosylation sites and three tyrosines in the cytoplasmic domain, with one of them within a classical ITSM sequence (59). There are two known transcripts, one of which lacks exon 5 containing a pseudo-like ITSM (59). Murine Cs1 contains a putative SH2-binding site but does not associate with SAP, EAT-2, or ERT, suggesting the existence of other adaptors (59, 60). In humans, however, one isoform does bind SAP and EAT-2 and recruits other downstream molecules such as Fyn for phosphorylation of its cytoplasmic tail (59, 61). Functionally, cross-linking of human Cs1 with an antibody activates NK mediated cytotoxicity via PI3K, PLCγ, and ERK-mediated

pathways (57, 58). Overall, functional characterization of this molecule has primarily focused on its role in human NK cells and is limited in mice. The generation of a knockout would greatly assist in elucidating the murine functions of this molecule.

CD48 (Blast-1, BDM1, sgp-60, Ox-45, BCM1)

CD48 was discovered by several groups as a glycophosphatidylinositol (GPI)-anchored protein whose expression is restricted to lymphocyte, macrophages, and dendritic cells (62-67). It is unique from all other SLAM family members in that it lacks a cytoplasmic tail, and is thus sometimes not considered a member of this family. It preferentially binds 2B4, but can also associate with CD2 at a lower affinity (64, 68). Generation of CD48 deficient mice showed that the functional defect was primarily localized to the T cell compartment. There was a small increase in CD4 T cells as well as increased CD2+ T cells. Functionally, these T cells show a reduced capacity to proliferate and produce IL-2 in response to ConA and anti-CD3 stimulation *in vitro* (62). These mice were completely defective in their ability to respond to PHA but responded normally to LPS (62). Interestingly, CD48 is the receptor for FimH, a component of the filamentous appendage on bacteria such as *E. coli*, suggesting that members of this family could potentially be involved in recognizing bacterial pathogens (69).

SLAM (CD150, IPO-3, CDw150)

SLAM, the founding member of the family, is expressed on CD4 and CD8 T cells, B cells, $CD11b^{+}$ macrophages, monocytes, and platelets, but not on NK cells (70-73). It has been identified as one of the co-receptors for the measles virus, and besides CD48, it is the only

other family member that has been identified to bind pathogens (74). Recently, it has been identified as a marker of long-term reconstituting hematopoietic stem cells that are fully capable of generating multiple lineages of blood cells (33). This function of SLAM could be mediated by the putative binding site for the PU.1 transcription factor in the promoter region which has been known to direct long-term engraftment of these cells (75, 76).

Like most members of this family, SLAM has two Ig-like extracellular domains and binds to itself at a low affinity (75, 77). There are two isoforms present in mice that differ only in their cytoplasmic tails. Isoform 1 has three ITSMs, whereas isoform 2 has only the most membrane proximal motif (73). A basal level of tyrosine phosphorylation in these motifs allows for SAP binding, while a fully phosphorylated state promotes competition among adaptors such as SHP-1, SHP-2, and SAP (53, 73, 78, 79). Recruitment of these downstream adaptors activates ERK and Akt pathways in both B and T cells (80, 81).

Based on studies using antibody-mediated engagement *in vitro*, SLAM was identified as a costimulatory receptor for T cells leading to increased production of T_H1 cytokines such as IFNγ and TNFα, without affecting T_H2 cytokine production (73, 82). However, mice deficient in this molecule exhibited a profound defect in IL-4 secretion with minimal effects on IFNγ production, and likely mediated by SAP and Fyn, given that deficiencies in these adaptors have similar defects (83-86). SLAM deficient macrophages also have decreased cytokine production when stimulated (83). There are no published reports on the B cell phenotypes in the knockout, although previously SLAM has been shown to enhance

proliferation and Ig synthesis but not class switching (80, 87).

Although SLAM has not yet been studied in SLE, a study recently demonstrated differences in SLAM expression in chronic inflammatory diseases such as Crohn's and multiple sclerosis (MS). Patients with Crohn's disease had increased numbers of SLAM positive cells throughout the inflamed gut whereas healthy controls showed expression present predominantly in the Peyer's patches. MS patients, on the other hand, did not show increased SLAM expression on monocytes present in the brain lesions suggesting that SLAM is not simply a marker of chronic inflammation (71). It would be interesting to determine the structural and/or functional polymorphisms in SLAM were mediating the chronic inflammatory responses seen in Crohn's disease.

CD84

CD84 is expressed ubiquitously in cells of the immune system, including platelets, B cells, T cells, and monocytes/macrophages, and, in mice, NK cells (49, 88). It contains three potential N-linked glycosylation sites in the two Ig-like extracellular domains with the first Ig domain mediating homotypic binding (49, 89). The cytoplasmic tail contains four tyrosines, two of which present in the TxYxxV/I motif, and can bind SAP and EAT-2 but not ERT (60, 88, 89). Functionally, human T cells stimulated with anti-CD3 and anti-CD84 antibodies have been shown to enhance proliferation and IFNγ production (49, 90). When human memory B cells were stimulated, a subset of cells expressing high levels of CD84 proliferated more rapidly to all B cell stimuli examined except for stimulation with CD40L

plus IL-4 (88). Furthermore, like Ly9, CD84 has been shown to have increased expression in T_{FH} cells in both mice and humans (55). Currently, there is very little known about the function of this molecule in mice.

Ly108 (NTB-A, SF2000, SLAMF6)

Ly108 is the most distal member of the family located in the *Sle1b* region, and is expressed on NK, T and B cells (91). There are three isoforms that differ solely in their cytoplasmic tails. Ly108-1 and Ly108-3 share the same exons, except that isoform 3 splices to a site upstream of exon 8, leading to the addition of 14 nucleotides (N. Limaye, unpublished observations). While Ly108-3 expressed at the RNA level, little else The two Ig-like domains contains nine potential N-linked glycosylation sites, and the cytoplasmic tail of isoform 1 and isoform 2 having two and three ITSMs, respectively (92, 93). It binds homotypically at a low affinity and does not appear to bind to any of the other family members in the *Sle1b* region (91, 94-96). In human NK cells, Ly108 constitutively associates with SHP-1, but upon treatment with pervanadate, SAP binds preferentially. However, in Jurkat T cells Ly108 does not associate with SHP-1 but can bind SAP (91, 95).

In vitro studies using anti-Ly108 specific antibodies and Ly108-Fc proteins on human NK cells show that engagement of Ly108 leads to increased IFNγ and TNFα production as well as enhanced NK-mediated cell lysis (91, 94, 96). These events are regulated by SAP as the loss of SAP leads to an inhibitory signal when Ly108 is engaged (91). In murine T cells, Ly108 acts in a costimulatory capacity with CD3 causing increased T cell proliferation and

IFNγ production, similar to anti-CD3 plus anti-CD28 stimulation, suggesting effects on T_H1 cells. There was no effect on T_H2 cytokine production *in vitro* (95). However, *in vivo* administration of Ly108-Fc suppressed T_H1 responses in T-dependent immunizations, and the fusion protein delayed disease onset of experimental allergic encephalomyelitis (EAE) (95). This discrepancy in T_H1 responses may be attributable to the fusion protein blocking Ly108 from binding its endogenous ligand *in vivo*, whereas the Ly108 antibody used *in vitro* would likely cross-link the receptor.

A Ly108-deficient strain has been generated, although a transcript containing the transmembrane and cytoplasmic domains remains that could potentially serve as a dominant negative protein (97). While there was no defect in IFNγ production in stimulated CD4 T cells, these Ly108-deficient cells did have decreased IL-4 and IL-13 production when stimulated with either anti-CD3 plus anti-CD28 or PMA plus ionomycin. This cytokine decrease was not restored with secondary stimulation (97). In addition to its function in T cells, Ly108 also plays a role in innate immunity since Ly108-deficient mice were more susceptible to *Salmonella typhimurium* infection even though they had elevated serum levels of IL-12p40, TNFα, and IL-6. This enhanced susceptibility was attributed to a neutrophil defect in reactive oxygen species (ROS) production, and not phagocytosis (97).

Amongst the genes within the SLAM family, Ly108 is the single most prominent candidate for mediating the *Sle1b* phenotype. There is significant expression difference in the ratio of isoform 2 to 1 in young mice prior to the onset of ANA, suggesting an inherent change and

not the effect of disease (27). CD4 T cells from B6.*Sle1b* mice exhibit the same defect in IL-4 production as that seen in the Ly108 knockout suggesting that the isoform utilization may be impacting T cell function. More recently, it has been suggested that the two Ly108 isoforms may have different functions in apoptosis and BCR signaling in immature B cells which may lead to alterations in B-cell tolerance (K.R. Kumar, *submitted*). Taken together, these data further support the notion of Ly108 as a causative gene.

SLAM FAMILY SPECIFIC DOWNSTREAM ADAPTORS

SLAM family members have ITSMs that can recruit proteins containing SH2-domains. The known SLAM family specific downstream adaptors include SAP, Eat-2, and ERT, all of which contain a single SH2-domain and a short C-terminal tail.

SLAM-Associated Protein (SAP, SH2D1A)

SAP is located on the X-chromosome in both mice and humans. Deficiency of this protein in human patients causes a disorder known as X-linked Lymphoproliferative disease (XLP). These patients have an uncontrollable lymphoproliferative diseases following an infection with Epstein-Barr Virus (EBV) (78, 98). SAP is expressed primarily in T cells, NK cells, and NKT cells, and it has been reported that a small percentage of B cells may also be expressing SAP (79, 99-101). Structurally, it contains a short tail as well as a SH2 domain that mediates binding to the TxYxxV/I motifs present in all SLAM family members, with the exception of CD48. SAP also been shown to serve as a linker between SLAM family members and FynT (102). Analysis of the crystal structure of SAP and various mutants

revealed that an arginine at position 78 is critical for SAP to interact with a non-canonical binding region in the SH3 domain of Fyn. This could activate the Fyn kinase domain (103, 104). Fyn can then phosphorylate other molecules including members of the SLAM family.

Studies from both SAP knockout mice and XLP patients have elucidated functional roles for SAP in the immune system. SAP deficiency does not affect the development of B, T and NK cells (84, 85, 105). However, mice deficient in SAP do exhibit a complete loss of NKT cells that is also observed in XLP patients (99, 106, 107). In addition to its role in NKT cell development, the most well characterized function of SAP is its ability to regulate T_H2 differentiation. When T cells are stimulated, SAP recruits Fyn and signals through the NFκB pathway to mediate T_H2 cytokine production (86, 108). Specifically, overexpression of SAP in T cells not only led to increased IL-4 production, but also a decrease in IFNγ production (103). However, examination of the CD4 T cell subset did not show a change in IFNγ production. SAP's role as a negative regulator in CD8 T cells might mediate the IFNγ effect (105, 109). Immunizations studies were also performed in SAP deficient mice and, as expected, showed a defect in T-dependent responses, but these mice had normal T-independent responses, suggesting that B cell functions are intact (110). However, antigen specific antibody generation were impaired when immunizations were performed in adoptive transfers experiments using Rag2-/- mice with SAP sufficient T cells and SAP deficient B cells (101). This supports a role for SAP in B cell function. Furthermore, B cells from SAP knockout mice produced less IgG2c, IgG2b, IgG3, and IgA when stimulated *in vitro* (100). Although SAP transcripts have not been readily detectable in bulk B cells, the data suggest

that the deficiency does appear to impact intrinsic B cell functions, perhaps in later B cell differentiation stages.

SAP also plays a critical role in controlling the immune response during infection. It has been demonstrated that SAP deficient mice infected with lymphocytic choriomeningitis virus (LCMV), or murine gammaherpesvirus-68 (MHV-68), the murine equivalent of EBV, led to robust T cell responses including increased IFNγ production. However, these mice were unable to effectively clear the viral infection (84, 85, 105, 109). The phenotype was not specific for viral responses as it was also seen in *Toxoplasma gondii,* an infection mediated by a parasite. Failure to control the immune response during these infections is attributed to the inability to establish antigen-specific long lived plasma cells in the absence of SAP (111).

SAP function has also been examined in the context of both an induced and spontaneous model of murine lupus. In the pristane-induced model of lupus, it was shown that SAP deficiency protected the mice from developing GN as well as caused a reduction in ANA levels (110). In the MRL/*lpr* model, mice that had inherited a spontaneous mutation in SAP during the breeding of the strain, leading to its deficiency, had a partial amelioration of disease. However, unlike the pristine model, these mice had the capacity to generate high titer IgG ANAs but not IgM ANA (112). Although SAP deficiency ameliorated the pathogenic processes of SLE, it has been shown that in the absence of SAP disease was exacerbated in the experimental autoimmune encephalitis (EAE) model of MS (110).

Eat-2 (*SH2D1B*, Eat-2a)

Eat-2, as well as ERT, are located on mouse chromosome 1 proximal to the SLAM family members (113, 114). In mice, Eat-2 has been shown to be expressed primarily in cells of the innate arm of the immune system, including macrophages, dendritic cells and NK cells, and has not been detected in splenic T or B cells (60, 93, 114, 115). Sequence analysis of this gene in a panel of inbred strains revealed no polymorphic differences among B6, BALB/c, 129, NZW and NZB, while there was an amino acid change from threonine to serine in NOD mice. Structurally, the protein encodes a single SH2 domain and a tail containing two tyrosine-based motifs (60, 113, 114). Eat-2 does not bind Fyn unlike SAP but has been shown to associate with 2B4 and not Cs1 or CD84 in murine NK cells (60, 103, 104).

The recent generation of both Eat-2 transgenic and knockout mice revealed that it functions as a negative regulator of NK cell function. The deficiency of this molecule led to enhanced NK cell cytotoxicity, whereas overexpression decreased the NK killing capacity (60). The functionality of this molecule is mediated by the two additional tyrosine motifs in the tail and not by the SH2 domain (60). Eat-2 modulates this response by inhibiting early tyrosine phosphorylation events during the activation of NK cell signaling (60). It remains to be determined how this molecule functions in other immune cell types, and what its role is in signaling for other SLAM family members.

ERT (*SH2D1C*, Eat-2b)

ERT is expressed abundantly in NK cells and at lower level in CD8 T cells and macrophages

(60, 114). It is believed to have arisen from a gene duplication event of Eat-2 on chromosome 1, and, in humans, it has further evolved in to a pseudogene (60, 114). Similar to Eat-2, the SH2 domain in ERT binds 2B4 and not CD84 or Cs1; and the tail contains two tyrosine-based motifs (60). However, ERT also contains a putative SH3-domain binding motif in the third exon. This domain does not interact with Src kinases but could potentially recruit other SH3-containing proteins (114). Based on the generation of the ERT knockout, it is functionally indistinguishable from Eat-2 and plays a role in negatively regulating NK cell function but does not impact NK cell development (60).

Sequence analysis of ERT revealed two polymorphisms between the B6 and 129 mouse strains, one of which encodes an amino acid change (114). Interestingly, the genomic location of ERT, in addition to Eat-2, maps to another SLE susceptibility locus known as *Sle1a*, which is upstream of the *Sle1b* region. These two loci, *Sle1a* and *Sle1b*, epistatically interact with each other and exacerbate the phenotype seen in B6.*Sle1b* mice (26). It is possible that the SNP present in the 129 allele of ERT may be interacting with the SLAM family haplotype in B6.*Sle1b* mice to further aggravate autoimmunity, although more analysis will need to be conducted.

CLINICAL SIGNIFICANCE FOR UNDERSTANDING ANA POSITIVITY

It is well established that autoantibody production precedes the development of clinical disease (3). This phenomenon is seen not only in SLE but also in other autoimmune diseases. For example, individuals positive for rheumatoid factor (RF), an autoantibody to

the Fc portion of IgG antibodies, or anti-cyclic citrullinated peptide (anti-CCP) are more likely to develop RA (116-118). In type 1 diabetes, approximately 70-80% of individuals with antibodies to diabetes-associated antigens will progress towards disease (119). Clearly, the presence of autoantibodies not only represents an initial biomarker but also marks the beginning of a discernible autoreactive immune response. This early autoimmune state provides an opportunity to intervene therapeutically prior to the development of irreversible clinical pathology. Thus, it is important to identify the causative genes and understand the pathways these genes are involved in to mediate the initial step towards autoimmune disease.

OBJECTIVES

We have identified a family of genes, the SLAM family, which can contribute to the generation of anti-nuclear autoantibodies. There are two objectives that will be discussed in the following chapters. First, we describe the identification of the 2B4 gene duplication, a member of this SLAM family. Second, we discuss the generation of various mouse models to dissect the role of the SLAM family in mediating the breach in tolerance to nuclear antigens. Both the identification of additional genes in the family, as well as understanding the mechanisms by which polymorphisms in this family play a role in mediating ANA production will potentially lead to the development of therapeutic targets that could be used to prevent the onset of SLE and other systemic autoimmune diseases.

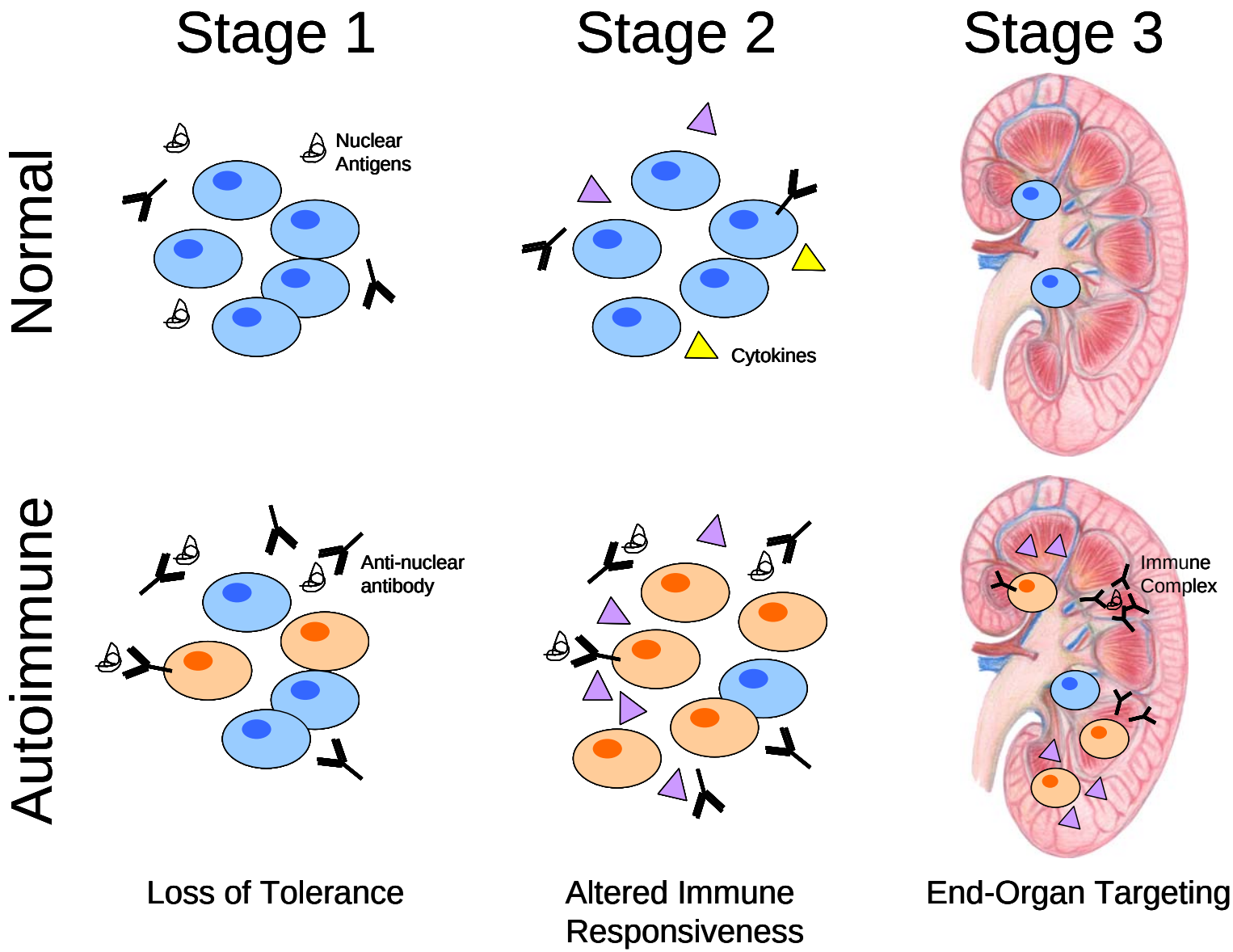


Figure 1. A Model depicting the three stages in Autoimmune Pathogenesis.

There are three stages in the development of autoimmune pathogenesis based on component phenotypes present in

susceptibility intervals. The first step is mediated by genes breaching tolerance to nuclear antigens, such as *Sle1*. Normally, the immune system is tolerized to self-antigens, although these antigens are present ubiquitously. In the second stage, these causative genes dysregulate the immune response by altering immune cell function or properties, such as cytokine production. Lastly, genes in stage three are involved in modifying inflammatory responses that mediate tissue destruction in end-organ targeting.

Member	Number of Northern Blot Transcript Bands (size)	Northern Blot Difference between B6 vs. Sle1b	B Cell Exp	T Cell Exp	Total SNPs		EC Domain SNP	
					N	S	N	S
2B4	2	ND	N/A	N/A				
Ly9	2 (3.2kb, 2.4kb)	Difference	Up	Down	7	5	6	4
Cs1	2 (3.1kb, 1.2kb)	No difference	Up	**Down**	2	0	0	0
CD48	1 (800bp)	No difference	Up	**Down**	2	0	1	0
SLAM	4 (8.9kb, 4.7kb, 2.3kb, 1.7kb)	No difference	Up	Down	0	2	0	1
CD84	3 (11kb, 3,1kb, 1,5kb)	No difference	**Up**	Up	1	3	1	0
Ly108	3 (4.7kb, 2.6kb, 1.5kb)	Difference	**Up**	**Down**	0	3	0	1

Table 1. SLAM family differences between B6 and B6.*Sle1b* mice.

Table 1 summaries expression and genetic differences between B6 and B6.*Sle1b* mice. Bold and underlined ‘up’ or ‘down’ indicate significant expression changes defined as two fold relative to B6 seen in B and CD4 T cells. The total SNPs in the gene are separated into non-synonymous (SNPs causing amino acid changes), N, and synonymous (SNPs conserving the same amino acid), S, mutations. Extracellular (EC) SNPs are also classified into the same categories. (ND, not determined; N/A, not applicable) (27)

Protein	Cell Type Expression	N-linked Glycosylation Sites	# Isoforms	# ITSM in each isoform	Isoform Differences	Adaptors
2B4	γδ T, NK, CD8, BA, MO	7	2	IsoL-4 IsoS-1	Cytoplasmic tail	SAP, ERT
Ly9	Thymocytes, mature B & T, bone marrow cells	8	2	Both have 2	Short isoform early start codon & missing IgV1 domain with 2 N-glycosylation sites	SAP
Cs1	NK, CD8, activated B, mature DC	5	2	Iso1-1 + 1 pseudo-like Iso2-1	One isoform lacks the pseudo-like ITSM	No known adaptors
CD48	Lymphocytes, Macs, DC	6	1	N/A	N/A	N/A
SLAM	CD4, CD8, B, Macs. MO, not NK	9	2	Iso1-3 Iso2-1	Cytoplasmic domain, shares 1 ITSM	SAP
CD84	all immune cells	3	possibly 2	2	Unknown	SAP, EAT-2
Ly108	NK, T, B	9	3	Iso1-2 Iso2-3 Iso3-ND	Cytoplasmic tails	SAP

Table 2. Summary of SLAM family features.

Abbreviations used in table: NK, natural killer; BA-basophils; MO-monocytes; DC-dendritic cells; Macs-Macrophages; Iso-isoform;

CHAPTER TWO

Materials and Methods

CONSTRUCTION OF THE 129-DERIVED BAC CONTIG

BACs were identified from both the 129Sv/J-derived BAC library (Invitrogen, Carlsbad, CA) and the 129Sv/Ev BAC library (BACPAC Resources, Oakland, CA) to span the *Sle1b* interval including the expanded 2B4 region. Markers used to generate the B6 BAC contig for this region were used to screen the 129 BAC library (27). BAC ends were sequenced using vector-specific primers from the BACPAC Resources website, and this sequence was used to generate additional markers to screen more BACs to span the 2B4 region.

MICE

The B6.*Sle1b* congenic strain contains an NZM2410-derived interval surrounding D1Mit113 (27). Two 129Sv/J(129)-derived congenic strains, designated as B6.129(113-115) and B6.129(113), were generated on the C57Bl/6 (B6) background by introgressing the 129 interval from D1Mit113 to D1Mit115 and D1Mit113, respectively. Using polymorphic markers spaced evenly throughout the genome, all 19 chromosomes were shown to have been derived from the B6 genome. BAC transgenic strains, generated as described below, were crossed on to either the B6 or B6.*Sle1b* strains. SAP knockout mice, generously provided by Pamela Schwartzberg, were bred to both the B6 and B6.*Sle1b* strains. All mice were housed under specific pathogen-free (SPF) conditions at the University of Texas

Southwestern Medical Center.

GENERATION OF BAC TRANSGENIC MICE

Bacterial Artificial Chromosomes (BACs), RPCI-23-438K9 containing the B6 allele of *Ly108* and RPCI-23-388C4 (BAC90) containing the B6 alleles of *Cd84* and *Ly108*, were identified from the B6-derived BAC library (BACPAC Resources, Oakland, CA). RPCI-22 350L6 containing the 129 allele of *Ly108* was selected from the 129Sv/Ev BAC library (BACPAC Resources, Oakland, CA). Two liter bacterial cultures from these BAC clones were grown in LB with chloramphenicol (12.5 μg/mL) overnight at 37^{o}. Qiagen Midi Kit solutions, P1, P2, and P3, were used to isolate BAC DNA (Qiagen, Inc., Valencia, CA). The DNA was then further purified by phenol-chloroform extraction followed by cesium chloride gradient ultracentrifugation. BAC DNA was then diluted to 50 ng/μL in TE buffer (10mM TrisHCl pH 7.5, 0.1mM EDTA) and injected into fertilized-oocytes derived from B6. Founder lines were identified by PCR specific for the vector ends and then bred on to respective genetic backgrounds.

SINGLE CELL SUSPENSIONS

Tissues were harvested and crushed using sterilized wire mesh in complete RPMI-10 media (10% fetal calf serum, 1% penicillin/streptomycin, 1% glutamine, 25 mM HEPES, and 10mM beta-mercaptoethanol). This tissue suspension was passed through an 18-gauge needle to generate a single cell suspension. Red blood cells were then lysed with ACK lysis buffer (0.1 mM EDTA, 0.15 M NH_4Cl, 10 mM $KHCO_3$, pH 7.2).

CELL PURIFICATION

Naïve $CD4^+$ T cells were purified by negative selection. Briefly, whole splenocyte suspension were incubated with biotinylated antibodies to B220 (RA3-6BB2), CD8 (53-6.7), NK1.1 (PK136), CD49b (DX5), HSA (M1/69), CD11b (M1/70), and Ter119 (BD Pharminigen, San Diego, CA). Naïve resting B cells were purified by negative selection using the following biotinylated antibody cocktail, CD43, CD4, CD8, NK1.1, Gr-1, CD11b, and Ter119 (eBioscience, San Diego, CA). Cells were then incubated with streptavidin-magnetic beads (BD Pharminigen, San Diego, CA) and placed on the Imagnet direct magnet (BD Pharmingen, San Diego, CA) according to manufacturer's protocol. Cell purity was greater than 90% based on flow cytometry analysis.

IN VITRO CELL STIMULATION

Cells purified by negative selection were plated at $1x10^5$ cells per well in complete RPMI-10 media. Naïve CD4 T cells were stimulated for 24 to 72 hours with plate-bound anti-CD3 (1 μg/mL) alone, or anti-CD3 plus anti-CD28 antibodies (10 μg/mL) (eBioscience, San Diego, CA). Anti-Ly108 (1 μg/mL), generously provided by John D. Schatzle, and anti-SLAM (1μg/mL) (Serotec, Raleigh, NC) were also added to the stimulation conditions for certain experiments.

RNA ISOLATION, CDNA PREPARATION, AND QUANTITATIVE REAL-TIME PCR

Total RNA was isolated using the Qiagen RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Inc., Valencia, CA). cDNA was then synthesized using oligo-dT in the Taqman Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was assayed using Sybr Green PCR Master Mix on the 7300 Real-Time PCR System according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA). Cycling conditions included an initial 10 minute denaturing step followed by 40 cycles of 20 seconds at 95 degrees, 20 seconds at the annealing temperature, and 30 seconds at 72 degrees. Primers used are listed in Table 3. All values were normalized to beta-2-microglobulin (β2M).

AUTOANTIBODY ELISAS

Sera were collected at 5, 7, 9, and 12 months. The detection of IgG autoantibodies against chromatin was performed as described previously (23). Briefly, Immunlux HB (Dynatech, Chantilly, VA) plates were precoated with 50 µl/well methylated BSA, followed by 50 µl/well of 50 µg/ml dsDNA (dissolved in PBS), and then 50 µl/well of 10 µg/ml total histones (Roche, Indianapolis, IN). Each coating step was for 30 minutes at 37^{o} and followed by two PBS washes. Plates were then incubated overnight at 4°C with 200 µl/well ELISA blocking buffer (PBS, 0.1% gelatin, 3% BSA, and 3 mM EDTA). Sera were added at a final dilution of 1/800 in serum diluent (PBS, 0.1% gelatin, 2% BSA, 3 mM EDTA, and 0.05% Tween 20) and incubated for 2 hours at room temperature. Bound IgG was detected using alkaline phosphatase-conjugated goat anti-mouse IgG (Chemicon) and *p*-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO) as the substrate. OD450 was measured by an

Elx800 Automated Microplate Reader (Bio-Tek Instruments, Winooski, VT) and the raw optical densities were converted to arbitrary normalized units using a six-point standard curve generated by a mAb derived from a NZM2410 mouse (120). A 1/250 dilution of this supernatant was set at 1000 U/ml.

MEASURMENTS OF CYTOKINE PRODUCTION

Supernatant from cell culture stimulations were assessed for cytokine quantity. IL-2 production was assessed using the BD OptEIA kit for mouse IL-2 according to manufacture's instructions (BD Bioscience, San Jose, CA). BD Cytometric Bead Arrays (BD Bioscience, San Jose, CA) were used for IFN-γ, IL-6, IL-4, IL-5, IL-10, IL-12p70, IL-13, and TNFα. Assays were performed according to the manufacturer's instructions and analyzed on the LSRII (BD Biosciences, San Jose, CA).

SNP-BASED ASSAY FOR TRANSGENE EXPRESSION AND COPY NUMBER DETERMINATION

The SNP-based assay was used to determine both transgene copy number as well as the expression of the transgene. Briefly, primers surrounding a SNP distinguishing the B6 and 129 or *Sle1b* allele were designed to amplify a 200-500 bp product. The PCR product was then subjected to ExoI and Shrimp Alkaline Phosphatase treatment to remove residual primers and primer dimers. The cleaned product was then used as a template for a single base extension PCR amplification using a primer that was positioned one base pair upstream of the SNP. Incorporation of the following nucleotide was dictated by the alleles that were

present in the template. This product was then loaded onto a CEQ 2000XL capillary sequencer (Beckman Coulter, Fullerton, CA) according to manufacturer's instructions. Data were analyzed on CEQ8000 Genetic Analysis System software (Beckman Coulter, Fullerton, CA). Transgene expression was assessed by the presence of two different color peaks, each corresponding to one allele. Transgene copy number was calculated based on the height of the peak normalized to the height of the peak obtained from heterozygous mice known to express equal copies of both alleles.

Primer Name	Annealing Temperature	Sequence
β2M	55	F – GACCCTGGTCTTTCTGGTGCTT R – TCAGTATGTTCGGCTTCCCATT
CD84	55	F – GTCCAGCAGCCATGTACAGACACT R – GCCAACATCGGAATGAGAATAAGC
CD48	55	F – CTTGAAGAAAAACAATGGTGCACTT R – TTCACGCAGCACTCTCATGTAGT
Cs1 RT Ex5-6	55	F – GGAGAACGCAGACTATGACACAA R – TTGGTGCATCTTCTTCTGGTCTT
Ly108-1	60	F (B6) – CATTCCAGAGAGGCCGAATACTCTT F (1b) – CATTCCAGAGAGGCTGAATACTCTT R – GGATCCCATTGCCAGTTG
Ly108-2	60	F – CCGGCTATAACCAACCCATT R – AAGCCAGAGCTGTGGTGACA
Ly108-3	60	F- ACTCGTCCAATGCAGGAAAT R – GAGAAATGGGCTCTCTGGAA
Ly9	55	F – TCCTGCACCTTCACCCTAATCTG R – GGGTGTGGCTTCCATCGTATGTAT
SLAM RT Ex6-7	55	F – AGCCTGCCCCAGAGTCTGT R – GGCAGTGTCACACTGGCATAA

Table 3. Table of real-time PCR primers used for the SLAM family members

All primers used were at a final concentration of 0.264 μM except for Ly108-1. For Ly108-1, the final concentration for each forward primer was 0.132 μM and the reverse primer was 0.264 μM.

CHAPTER THREE

Mapping of the 2B4 Expansion in the *Sle1b* Haplotype

OVERVIEW

Congenic dissection and fine-mapping analysis led to the identification of a 940 kb region, termed *Sle1b*, as a murine autoantibody susceptibility locus. This region, near the telomeric end of mouse chromosome one, lies just distal to the Fc receptor cluster and is centered around the microsattelite marker D1MIT113. The most obvious candidate genes within this interval are members belonging to the SLAM family, due to their known roles in modulating immune cell function and responses. Seven members of this family, *2b4*, *Ly9*, *Cs1*, *Cd48*, *Slam*, *Cd84*, and *Ly108*, lie within the critical region for mediating the autoimmune phenotype. All the genes with the region were analyzed for sequence and expression differences, but structural and expression differences were primarily present in members of the family. The polymorphisms within the family formed a stable haplotype present in the majority of inbred mouse strains, including 129/SvJ (129). By introgressing this critical region from either 129 or the wild-derived inbred Cast/Ei strains onto the B6 background, we were able to recapitulate the loss in tolerance to chromatin seen in B6.*Sle1b*. This suggested that the same allelic variants within the interval that cause autoantibody production in B6.*Sle1b* are also present in 129 and Cast/Ei.

Preliminary analysis of polymorphisms within the extracellular domain of 2B4 suggested that

more than one copy of the gene existed in this region in strains carrying the *Sle1b* haplotype. Although *2b4* does not satisfy the necessary *Sle1b* candidacy criteria, *i.e.* expression in B or CD4 T cells, it is a member of the family and could potentially play an accessory role in mediating the phenotypic consequences of the actual disease-causing allele(s).

In this chapter, a characterization of the genomic expansion of the *2b4* gene will be presented. Based on the haplotype similarity between the NZW-derived *Sle1b* and 129, we used a 129 BAC library to generate a contiguous sequence (contig) spanning the expanded region. We also determined whether this expansion is seen in both inbred and wild-derived mouse strains. Lastly, we examined the autoimmune-prone *2b4* allele in comparison to the B6 allele in order to determine the candidacy of the gene for mediating ANA production.

RESULTS

Generation of a 129-derived BAC Contig

BACs from the CITB and RP22 libraries, derived from the 129Sv/J and 129SvEv genomes, respectively, were screened for those that contained genomic segments from the *Sle1b* interval. Using PCR primers previously used to map and order the B6-derived BACs from the RP23 library in this region (27), we were able to identify and array a total of 46 BACs (Figure 2).

For the 2b4 region, a marker designed in the extracellular domain of the *2b4* gene originally used for the generation of the B6 BAC contig, was used to array the BACs. Several positive

BACs were identified for the *2b4* marker; however, not a single BAC contained both the 5' and 3' flanking regions surrounding *2b4*. This suggested that the *2b4* region in 129 was larger than the corresponding region in B6 and was likely larger than the average BAC insert size. Moreover, previous sequencing of this *2b4* marker yielded multiple products suggesting that more than one copy of *2b4* is present in the 129 genome.

To identify the number of different *2b4* copies that were present in the 129 genome, the extracellular domain containing IgV and IgC were sequenced from the various 129 BACs positive for the *2b4* marker. Sequencing of the IgV and IgC domains revealed four unique sequences. Southern blot analysis of the positive BAC clones confirmed the existence of the different *2b4* genes, or isotypes, based on the different size fragments using a probe for the extracellular domain. A few BACs showed two bands indicative of the presence of two different *2b4* isotypes. To assist in ordering the remaining 2B4-positive BACs, isotype-specific primers were designed based on the sequencing data. BACs were screened and identified as carrying either isotype 1 and 2 or isotype 3 and 4. However, none of the BACs were positive for both isotype 2 and 3 leaving one gap between these two forms. In an attempt to close the final gap, BAC end sequencing was initiated to identify unique PCR products for that region that were not duplicated in the other BACs. One BAC end sequence from RP22-416B10 (called S416) identified a unique product that was not present in any other *2b4* containing BAC. Subsequent screening identified BACs positive for type 2 plus S416 or type 3 plus S416, thus closing the final gap between type 2 and 3. The final tiling path required five BACs to span the *2b4* duplication, which put the length of the expanded

interval at roughly estimated 400kb.

The *2b4* Gene Duplication is Present in Common Laboratory Inbred Strains and Wild-derived Mice

Based on the sequencing data identifying two stable haplotypes for the *Sle1b* region, we were interested in determining whether the expansion of *2b4* segregated with the existing two haplotypes (27, 29). Using PCR primers specific for the different *2b4* types, we screened genomic DNA from a panel of inbred and wild-inbred mice. Similar to the previously defined haplotype groups, the inbred strains could be divided primarily into two groups: one containing only *2b4* type 1 and the other having all four different types. The expansion correlated with the same inbred strains that contained the lupus-prone haplotype. Four of the inbred strains, however, had only types 2 through 4: AKR/J, DDY/Jcl, FVB/NJ, and R3/Dmmob. It may be possible that subtle and undetected mutations in *2b4* type 1 from these strains might have prevented their amplification considering the stringency of the *2b4* type-specific PCR conditions.

In contrast, the wild-derived strains carried various combinations of the different types. Some strains, such as Cast/Ei, did not amplify any of the *2b4* gene products but did amplify the non-type specific marker (*i.e.* original marker used in B6 BAC contig). It is possible that the different *2b4* versions had mutated significantly in the wild-derived strains leading to the difficulties in amplifying and detecting the various *2b4* PCR products. Table 4 summarizes the results from all the different strains tested.

2B4 is Most Likely Not Responsible for the ANA Phenotype in *Sle1b*

Due to the expansion of the 2B4 gene only in the haplotype that is permissive for lupus on the appropriate background, it is a possible candidate for the causative gene, although, as alluded to earlier, it does not meet the cell-specific expression criteria, *i.e.* expression in B or CD4 T cells (27). To assess its candidacy, we compared the protein sequence of the extracellular domains from the lupus-prone haplotype, to B6. Since we have shown that introgressing the haplotype from 129 or Cast/Ei onto the B6 genetic background is sufficient to mediate autoantibody production, we limited our comparison with B6 to these two strains.

Protein alignments of the extracellular domain from the different types of *2b4* from 129-derived BACs uncovered an early termination codon in type 3 (Figure 3). Cloning and sequencing of the IgV and IgC domains from splenocyte cDNA suggested that type 4 was the predominant type expressed in 129 mice. Thus, it explained the allelic forms that had been previously described (121, 122). Interestingly, sequencing of the IgV and IgC regions from Cast/Ei revealed that only one version of *2b4* was present, unlike other strains possessing the remainder of the autoimmune-permissive SLAM haplotype. Using the ClustalW alignment program, the *2b4* from Cast/Ei had the greatest similarity to the single B6 *2b4* product, and least identity with the predominant *2b4* type expressed in 129 (Figure 3, Figure 4) (123). This suggested that *2b4* is most likely not mediating the ANA phenotype in B6.*Sle1b* mice.

SUMMARY

In this chapter, we described the identification of a panel of 129-derived BACs that span the *Sle1b* critical interval. The elucidation of this 129 BAC contig serves two critical functions. Firstly, we identified BACs carrying the *Sle1b* causative allele(s), since the equivalent *Sle1b* interval from 129 is capable of breaching tolerance at the same penetrance as B6.*Sle1b*. Importantly, since there is currently no NZW-derived BAC library, these 129 BACs provide us with the tools for manipulating the genome *via* transgenic introduction of disease causing alleles. Secondly, we were able to define the genomic region containing the *2b4* expansion. We determined that the lupus-prone haplotype contains four unique *2b4* genes. Type 3 is presumed to be a pseudogene, due to the presence of an early termination codon in the middle of the IgV extracellular domain. Although type 1 is most similar to B6, type 4 is the predominant form expressed in splenic cDNA. These data corroborate and explain the allelic forms of *2b4* that have been previously described (121, 122). Our analysis is this study was limited to the extracellular domain due to difficulties sequencing the full-length cDNA from the various *2b4* types.

Analysis of inbred strains for the different types of *2b4* divides them into two haplotype groups that correlate with the previously described haplotype division for the other family members (27, 29). The majority of inbred strains belonged to the group having all four copies, similar to *Sle1b*, while the second group, like B6, contained only one copy. There were, however, a few exceptions: AKR/J, DDY/Jcl, FVB/NJ, and RIII. Wild-derived strains exhibited a more complex pattern most likely attributed to further diversification, perhaps due to selective pressures.

One particular strain, Cast/Ei from the wild-derived panel, was noteworthy due its ability to mediate autoimmunity when the genomic region equivalent to *Sle1b* is introgressed on to the B6 background. Analysis of *2b4* in Cast/Ei showed that only one copy was present. Protein alignment and phylogenetic analysis showed that 2B4 from Cast/Ei was most similar to B6 and not to the predominant version expressed in both 129 and B6.*Sle1b*. Taken together, these data suggest that the *2b4* expansion is not necessary for the development of the ANA phenotype, thus eliminating one SLAM family gene and leaving the other six members as strong candidates.

129-derived BAC Library	Clones	S48o11	USF1	T81k15	T116m12	ESTM33	D1Sta1	2B4 50kb	2B4-1	2B4-2	S416	2B4-3	2B4-4	T164 j21	LY-9	S116m12	T250o12	FLE2	S170o18	T330p8	CD48	S29a12	T73f1	S250o12	T170o18	S361k16	SLAM	S270h2	1-113	S330p8	T29a12	T270h2	T559k12	B18S	S558p15	B17S	S77a8	Ly108-2	Ly108-1	B15U	B2U	B18U	D1Sta2	B17U	NHLH1	PXF	T112i2	B16U	B2S	B15S
CITB	1 G9	-	+	+	+	+	+	+	-	-		-	-																																					
CITB	2 J9	-	+	+	+	+	+	+	-	-		-	-																																					
CITB	2 J10	-	+	+	+	+	+	+	-	-		-	-																																					
CITB	13 I18	-	-	+	+	+	+	+	-	-		-	-																																					
CITB	13 J17	-	-	+	+	+	+	+	-	-		-	-																																					
CITB	159 L1	-	-	-	+	+	+	+	+	-	-	-	-	-	-																																			
RP22	534 D20					+	+	+	+	-	-	-	-	-	-	-																																		
RP22	509 M1					-	+	+	+	-	-	-	-	-	-	-																																		
RP22	533 D1					-	+	+	+	-	-	-	-	-	-	-																																		
RP22	465 B20								+	+	-	-	-																																					
RP22	489 G18					-	-	-	-	+	+	-	-	-	-	-																																		
RP22	402 H11								-	+	+	-	-																																					
RP22	461 F7								-	+	+	-	-																																					
RP22	461 H7								-	+	+	-	-																																					
RP22	471 B4								-	+	+	-	-																																					
RP22	338 D23								-	-	+	+	-																																					
RP22	384 D17								-	-	+	+	-																																					
RP22	435 A12								-	-	+	+	-																																					
RP22	416 B10								-	-	+	+	-																																					
RP22	329 L13								-	-	+	+	+																																					
RP22	524 O4					-	-	-	-	-	-	+	+	-	-	-																																		
RP22	293 A13								-	-	-	+	+																																					
RP22	494 I18					-	-	-	-	-	-	-	+	+	+	+																																		
RP22	495 J6					-	-	-	-	-	-	-	+	+	+	+																																		
RP22	536 F1					-	-	-	-	-	-	-	+	+	+	+																																		
RP22	538 O24					-	-	-	-	-	-	-	+	+	+	+																																		
RP22	576 D14					-	-	-	-	-	-	-	+	+	+	+																																		
CITB	342 I14					-	-	-	-	-	-	-	+	+	+	+	-	-	-																														
CITB	341 O23					-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-																							
CITB	549 O15					-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-																						
CITB	346 A16					-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-		-	-																	
CITB	346 B15					-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-		-	-																	
CITB	250 O12		-	-	-		-	-						-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-		-			-			-	-	-				
CITB	170 O18		-	-	-		-	-						-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-		-			-			-	-	-				
CITB	170 P18		-	-	-		-	-						-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-		-			-			-	-	-				
CITB	330 P8			-	-		-	-						-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-		-			-			-	-	-				
CITB	29 A12			-	-			-						-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-		-			-			-	-	-				
CITB	559 K12		-	-	-		-	-						-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-		-			-			-	-	-				
CITB	361 K16		-	-	-		-	-						-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-		-			-			-	-	-				
CITB	558 P15	-	-	-	-	-	-	-						-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	+	+	+	+	-	-		-		-			-	-	-	-			
CITB	286 M20							-						-	-																	-	-	+	+	+		+	+	+	+	+	+	+	+	+	-	-	-
CITB	367 A7							-	-	-		-	-	-	-																	-	-	-	-	+		+	+	-	-							
RP22	344 G20																													-		+	+	+	+	+	+	+	-	-	-							
RP22	345 K3																													-		-	+	+	+	+	+	+	-	-	-							
RP22	350 L6																													-		-	+	+	+	+	+	+	+	+	+							
RP22	428 G20																													+		+	+	+	+	+	+	+	-	-	-							

Figure 2. 129-derived BAC contig of the Sle1b interval

129-derived BACs from both the CITB and RP22 libraries were identified and ordered using primers initially generated for the B6-derived BAC contig (27). Additional primers were designed to help array the 129 BACs in the 2B4 region. Yellow boxes indicate positive product for that primer, blue boxes indicate negative product, and white boxes were undetermined. Columns indicate marker name. A total of 46 BACs were ordered across the interval.

A. Common Laboratory Inbred Strains

Strain	2B4 Types			
	1	2	3	4
129/SvJ	+	+	+	+
A/J	+	+	+	+
AKR/J	-	+	+	+
BALBC/J	+	+	+	+
C3H/HeJ	+	+	+	+
C57BL/6By	+	-	-	-
C57BL/6J	+	-	-	-
C57BR/cdj	+	-	-	-
C57L/J	+	-	-	-
CBA/J	+	+	+	+
CE/J	+	ND	+	+
DA/HUSN	+	+	+	+
DBA/2J	+	+	+	+
DDY/Jcl	-	+	+	+
FL/Ire	+	+	+	+
FVB/NJ	-	+	+	+
HTG/Gosn	+	+	+	+
I/LnJ	+	-	-	-
LP/J	+	+	+	+
MRL/LpJ	+	+	+	+
NOD/Lt	+	+	+	+
NZB/BinJ	+	+	+	+
NZW	+	+	+	+
NZW/LacJ	+	+	+	+
P/J	+	+	+	+
PL/J	+	+	+	+
RF/J	+	-	-	-
RIII/dmmob	-	+	+	+
SB/Le	+	+	+	+
SEA/GnJ	+	+	+	+
SJL/Bm	+	+	+	+
SJL/J	+	+	+	+
SM/J	+	+	+	+
SWR/J	+	+	+	+
WB/Re	+	+	+	+

B. Wild-derived Outbred Strains

Strain	2B4 Types			
	1	2	3	4
BID	+	+	+	+
BIK	+	+	+	+
BZO	-	-	-	-
CIM	-	-	-	-
CTA	-	+	+	-
CTP	+	+	+	+
DDO	+	+	+	+
DEB	-	+	+	-
DGA	-	+	-	+
DHA	-	+	-	+
DLK	-	+	+	-
DJO	+	+	+	+
DMZ	+	+	+	+
DOT	+	+	+	+
KAK	-	+	-	-
MAC	+	+	+	+
MAM	+	-	-	-
MBK	-	+	-	+
MBS	-	+	-	-
MDH	-	-	-	+
MGA	+	+	-	+
MOL	-	+	-	+
MPB	-	+	+	+
MPR	-	+	+	+
SEB	-	-	-	-
SEG	+	-	-	-
SFM	-	+	-	+
SMZ	-	-	-	+
STF	+	-	-	-
THE	-	-	-	-
XBS	-	-	-	-
ZRU	-	-	-	-
22MO	+	+	+	+

C. Wild-Derived Inbred Strains

CASA/RK	+	+	-	-
CALB/RK	-	-	-	-
CAST/Ei	-	-	-	-
MOLC/RK	-	-	+	-
MOLD/RK	+	-	+	-
MOLE/RK	-	+	-	-
MOLF/Ei	-	+	-	-
Mus Pahari	-	-	-	-
PERA/Ei	+	+	+	+
PERA/RK	+	+	+	+
PERC/Ei	+	+	+	+
SF/CamEi	+	+	+	+
SK/CamEi	+	+	+	+
SK/CamRK	+	+	+	+
CZECHI/Ei	-	+	+	+

Table 4(A-C). Analysis of 2B4 types in inbred and wild-derived strains.

The table identifies the presence of the different types of 2B4 in (A) common laboratory inbred strains, (B) wild-derived outbred strains, (C) wild-derived inbred strains.

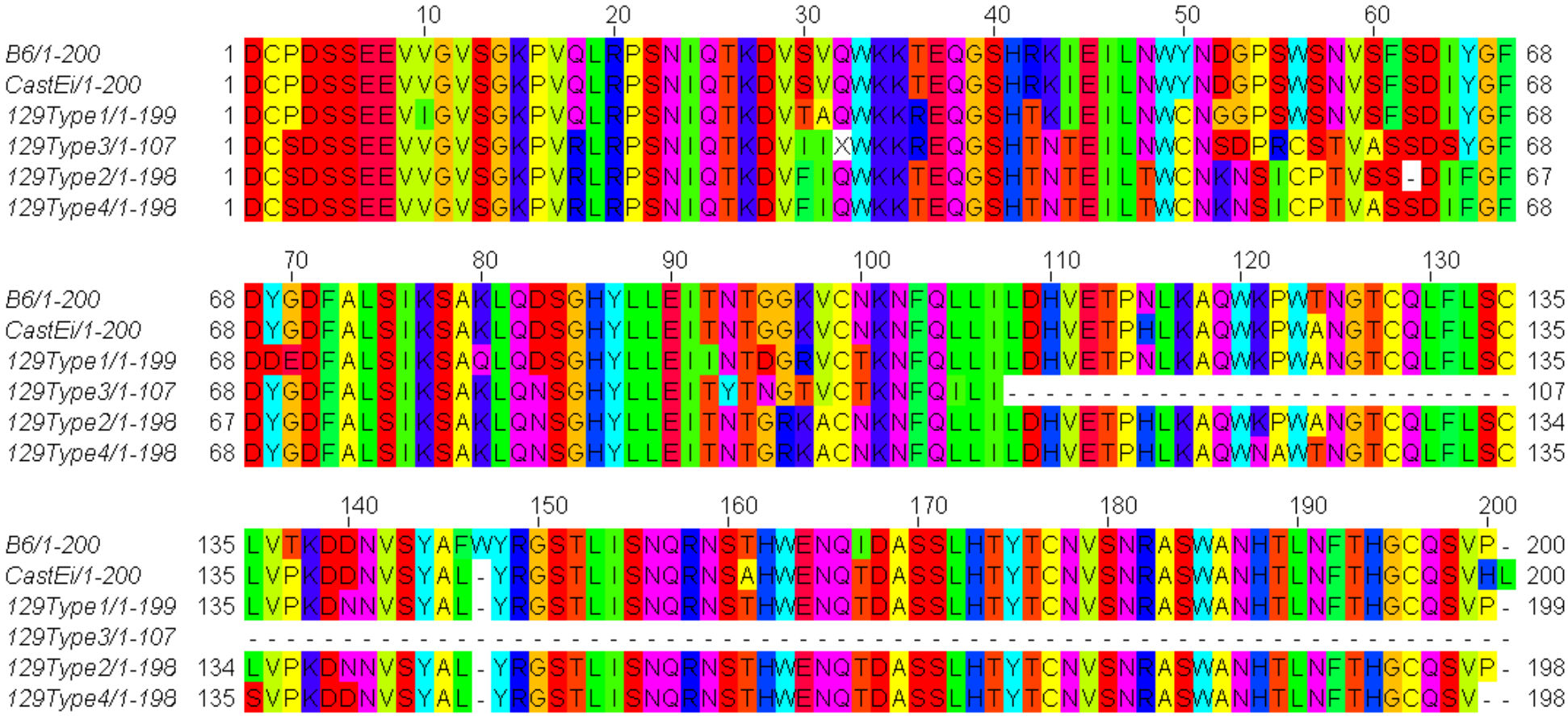


Figure 3. Protein alignment of the extracellular domains of 2B4.

The extracellular domains of 2B4 from B6, CastE1, and 129 were compared using the Jalview program (124). Protein alignment shows greater similarity between B6 and CastEi than the other types of 2B4 present in 129. The "X" at position 32 in 129 Type 3 represents the early termination codon.

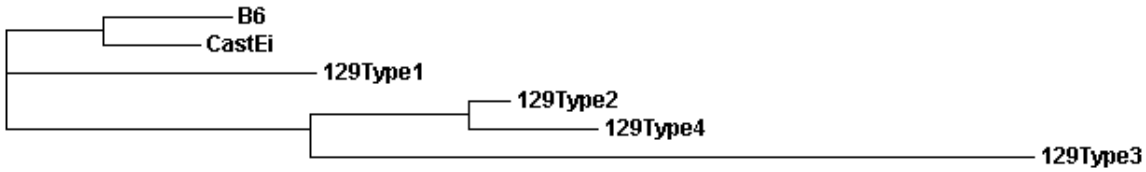


Figure 4. Phylogram tree of the extracellular domains of 2B4.

This phylogram tree was generated using the ClustalW program (123). It depicts the degree of sequence similarity between the different types of 2B4 genes present in B6, Cast/Ei, and 129. Clearly, the single *2b4* gene products in B6 and Cast/Ei have more similarity to each other than to remaining *2b4* forms found in 129. Type 3 from 129 is most distant due to the presence of the early termination codon.

CHAPTER FOUR

Identifying the Causative Allele for *Sle1b* Through the Generation of Mouse Models

OVERVIEW

Our analyses thus far have provided two strong pieces of evidence that support the idea that the SLAM family is causative for the loss in tolerance to chromatin seen in B6.*Sle1b* mice. First, we have firmly established that the extensive number of polymorphisms in the SLAM family seen in B6.*Sle1b* form a stable haplotype based on genomic analysis of a panel of inbred mouse strains. This haplotype, when placed in the context of the B6 genome, regardless of whether derived from 129 or Cast/Ei was sufficient to mediate a breach in tolerance to nuclear antigens. Second, candidate gene expression differences further support SLAM family member(s) as being causal because only these genes showed statistically significant expression differences between B6 and B6.*Sle1b*. However, we have yet to determine whether the phenotype is mediated by the polymorphisms in the entire family, a combination of alleles of specific members, or a single member of the family.

To identify the causative allele, we utilized a BAC transgenic rescue approach whereby a panel of B6-derived BACs spanning the *Sle1b* interval was used to generate a series of transgenic mouse lines. This strategy was based on the fact that mice heterozygous at the *Sle1b* locus have a severe reduction in ANA penetrance, and thus exogenous introduction of a B6 allele onto a mouse homozygous for *Sle1b* would lead to suppression only if the BAC

harbored the causal gene. In previous studies, five out of six BACs, spanning the *Sle1b* interval from candidates genes *Usp23* to *Pxf*, were generated and assessed for suppression of autoantibody production at 9 months (29). However, none were able to significantly suppress the ANA phenotype, thus eliminating 17 genes from the interval as causal genes. The phenotype of the BAC transgenic carrying *Cd84* and *Ly108* remained to be determined.

Although the identification of the causative gene(s) has remained elusive, this chapter provides insight into its identity through the generation of different BAC transgenic mice. Here we describe the final completion of the BAC transgenic rescue studies, and identify the 129 allele of *Ly108* as one of the causative genes for autoantibody production within the *Sle1b* interval.

RESULTS

Introduction of the BAC Carrying B6 alleles of *Cd84* and *Ly108* Suppresses ANA Production in B6.*Sle1b*

To complete the BAC transgenic rescue study, we performed pronuclear injections of B6 fertilized eggs with BAC90, which carries the B6 alleles of *Cd84* and *Ly108*. From the seventeen potential founders obtained, a total of twelve provided germline-transmission of the BAC90 transgene. Using these founders, the BAC90 transgene was moved onto the B6.*Sle1b* background using marker-assisted selection.

We utilized a sequencing-based SNP assay to determine that the B6 alleles of *Cd84* and

Ly108 were expressed in three of the founder lines, termed 4270, 4389, and 4390. Line 4390 had the highest transgene expression levels, and line 4389 had the lowest. All three lines had approximately two copies of the BAC based on normalizing the ratio of the transgenic and endogenous peaks to a B6.*Sle1b* heterozygous mouse, which has equal copies of the B6 and *Sle1b* alleles.

The generation of the BAC90 transgenic completed the panel of mice needed to assess impact of the *Sle1b* candidate genes on ANA production (Figure 5). We analyzed ANA penetrance for all the BAC founder lines at 7 months of age. For each BAC line, there were at least two founders that were assessed for autoantibody production. Negative littermate controls from all founder lines were pooled to determine the ANA penetrance of B6.*Sle1b* mice. Compared to negative littermate controls, only BAC90 reached statistical significance ($p<0.0001$) in suppressing the ANA production mediated by the *Sle1b* interval (Figure 6).

We also compared the mean ANA titers among all the BAC transgenic lines. BAC25 and BAC90 had mean titers statistically different from B6.*Sle1b* mice, $p<0.01$ and $p<0.001$, respectively (Figure 7). Although the mean titer of BAC25 was significantly different from B6.*Sle1b*, the ANA penetrance was comparable to B6.*Sle1b* suggesting that BAC25 does not contain the gene necessary to suppress the breach in tolerance. However, it does contain a gene (*2b4*, *Ly9*, and/or *Cs1*) that can impact the production of high titer ANA.

BAC90, on the other hand, was the only BAC able to suppress ANA penetrance and mean

titer, suggesting that *Cd84* or *Ly108*, or a combination of the two genes, mediates the loss in tolerance. BAC90 transgene had a reduced ANA penetrance of 26% (n=19, 2 founder lines), whereas negative control littermates had a 75% penetrance (n=4) (Figure 8, top panel). ANA titers in ANA negative BAC90 mice were comparable to B6 (Figure 8, bottom panel). Overall, these data strongly support that the B6 alleles of *Cd84* and/or *Ly108* can suppress the development of autoantibodies in B6.*Sle1b* mice.

Ly108 is a Causative Gene for Autoantibody Production

To determine whether *Ly108* alone was sufficient for mediating the suppression of ANAs seen in B6.*Sle1b* mice carrying the BAC90 transgene, a BAC transgenic mouse carrying only the B6 allele of *Ly108* (referred to as B6.*Ly108*-B6) was generated and bred onto B6.*Sle1b* as described above. Three founders underwent germline transmission and expressed the B6 allele of *Ly108* on the B6.*Sle1b* background, as ascertained by the SNP assay. B6.*Sle1b* mice have a characteristic *Ly108* isoform usage pattern differing significantly from B6 mice. B6 express more isoform 2 than isoform 1, thus giving a higher 2:1 ratio when compared to B6.*Sle1b*. As expected, the [B6 X B6.*Sle1b*]F1 mice have a 2:1 ratio intermediate to that of B6 and B6.*Sle1b*. We were interested in determining if the B6 allelic transgene on B6.*Sle1b* could change the 2:1 ratio and, if so, to what degrees. The transgene did indeed increase the Ly108 2:1 isoform ratio to a level intermediate between B6.*Sle1b* and a heterozygote (Figure 9). While none of the three lines fully recapitulated the level of expression of the B6 allele to that seen in a heterozygous mouse, the introduction of the B6 *Ly108* allele alone on the B6.*Sle1b* background was able to mediate suppression of autoantibody production from 75%

in B6.*Sle1b* (n=4) to 41% in B6.*Ly108*-B6 (n=22, 3 founder lines) at 7 months of age (Figure 10, top left panel). At 9 months the suppression, although not as dramatic, was still present, from 100% in B6.*Sle1b* (n=10) to 73% (n=15) (Figure 10, top right panel).

Since 129 and B6.*Sle1b* share the same SLAM family haplotype, and the congenics have similar ANA penetrance, we embarked on the reciprocal experiment by generating a BAC transgenic carrying the 129 allele of *Ly108* on the B6 background (termed B6.*Ly108*-129). Out of 13 potential founder lines, 10 transmitted the transgene, while nine actually expressed the 129 allele. The introduction of the 129 allele did lead to a decrease in the ratio of the two *Ly108* isoforms, though not to the extent seen in mice heterozygous at *Sle1b* (Figure 11). We selected four lines showing the most significant change. All four founder lines had an estimated one to two copies of the BAC, as determined by the SNP assay. As seen in Figure 12, B6.*Ly108*-129 mice had a 17% penetrance of ANAs compared to B6 mice which had none demonstrating a clear breach in tolerance. Consistent with the intermediate change in the *Ly108* isoform ratio, the penetrance and titer relative to B6.*Sle1b* heterozygotes were less than what would be expected in a transgenic line that recapitulated the heterozygote state.

SUMMARY

In order to determine which of the genes in the interval was responsible for the loss in tolerance to chromatin seen in B6.*Sle1b* mice, a BAC transgenic ‘rescue’ strategy was utilized. A series of B6.*Sle1b* transgenic mice, carrying B6-derived BACs spanning the *Sle1b* critical region, were created in order to interrogate *in vivo* which gene modulated ANA

production, and was able to best recapitulate the suppression observed in mice heterozygous at *Sle1b*. These analyses revealed that only one BAC in the *Sle1b* tiling path, BAC90, was able to mediate suppression. This BAC carries the two most distal genes of the SLAM family within the *Sle1b* interval, *Cd84* and *Ly108.* It is known that both these genes are homophilic members of the family, and have been shown to impact cytokine production, such as IFNγ, in T cells and other immune cell types. Furthermore, previous studies characterizing the expression and structural polymorphisms of the genes within the *Sle1b* interval indicated that *Ly108* was the best single gene candidate, due to the pronounced differential isoform usage seen between B6 and B6.*Sle1b*.

Based on these data, we wished to determine whether the different alleles of *Ly108* were able to mediate reciprocal effects on autoantibody production in the absence of any contribution from *Cd84*. To achieve this, we generated BACs carrying *Ly108* alone having either the 129 allele (*Ly108*-129) or the B6 allele (*Ly108*-B6) on the B6 or B6.*Sle1b* backgrounds, respectively. Using these approaches we were able to demonstrate that the 129 allele of *Ly108* was able to recapitulate the breach in tolerance seen in B6.*Sle1b*. In a reciprocal fashion, the introduction of the B6 *Ly108* allele on the B6.*Sle1b* resulted in intermediate suppression of the *Sle1b* phenotype. It is important to note that in both types of transgenic lines the exogenous introduction of the other allele did not completely restore the expression of the two *Ly108* isoforms to what is observed in B6.*Sle1b* heterozygotes. This correlates well with the partial modulation on autoantibody production. It is possible that if the 129 *Ly108* allele was introduced onto a Ly108 knockout background (generated in B6 ES cells),

then the isoform ratio may be fully recapitulated and the ANA production would reach similar levels to that seen in B6.*Sle1b*.

When ANA titers were assessed for all the BAC transgenic lines, both BAC25 and BAC90 had statistically lower mean ANA titers than B6.*Sle1b* mice, however, BAC25 did not suppress ANA penetrance. Thus, although BAC25 does not suppress the breach in tolerance, it does suppress high titer ANA. It is hence possible that the full *Sle1b* phenotype requires the combined contributions of *Ly108, Cd84* and allelic variants of genes within BAC25 (*2b4, Ly9*, and/or *Cs1*).

..

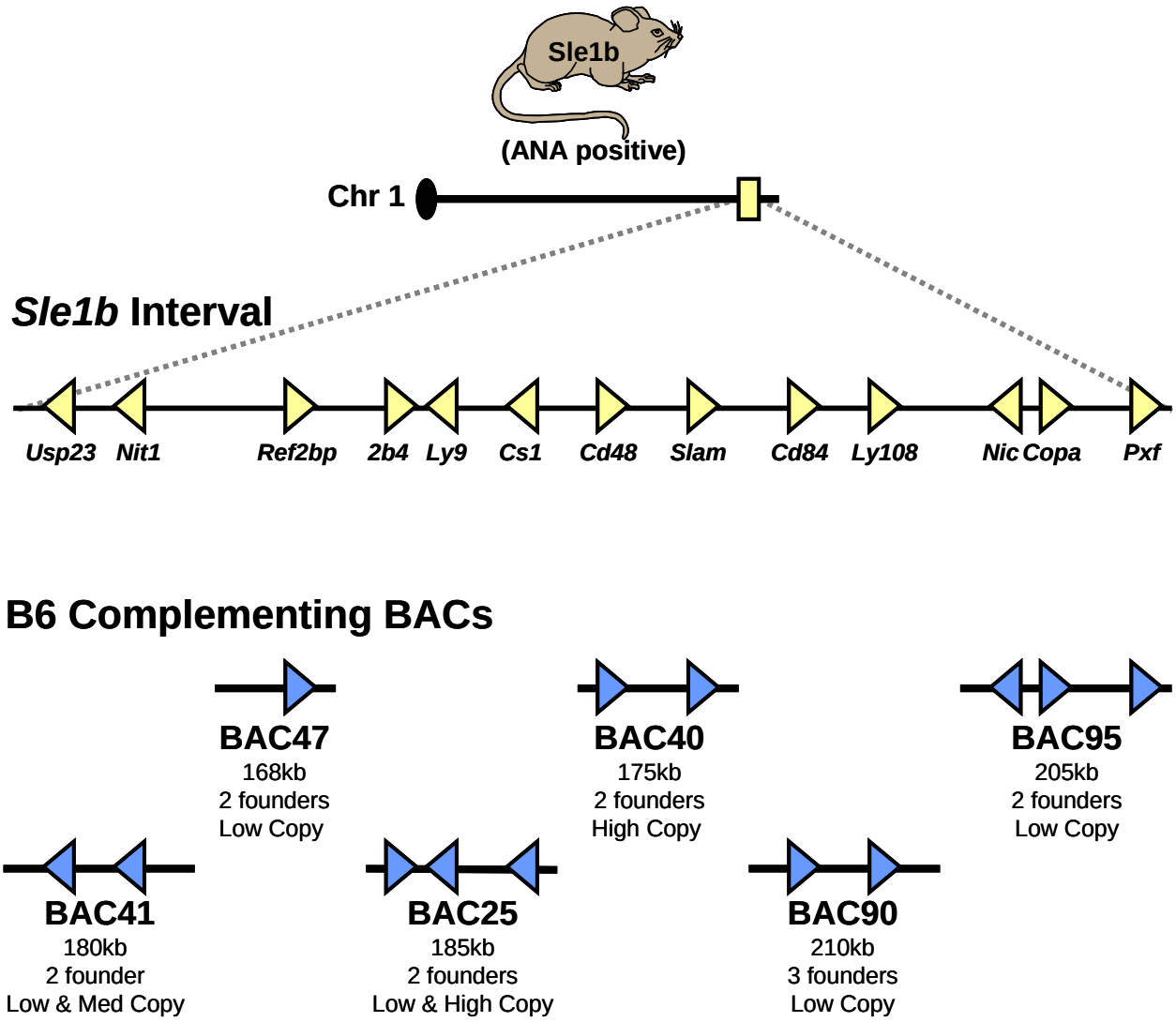


Figure 5. B6-derived BACs used in the BAC transgenic rescue approach.

This diagram illustrates the six B6-derived BACs spanning the *Sle1b* interval. Each BAC carries B6 alleles that genetically complement a region in the *Sle1b* interval. The characteristics of the transgenic lines are described (Low copy: 1-2; Med copy: 3-4; High copy: >10).

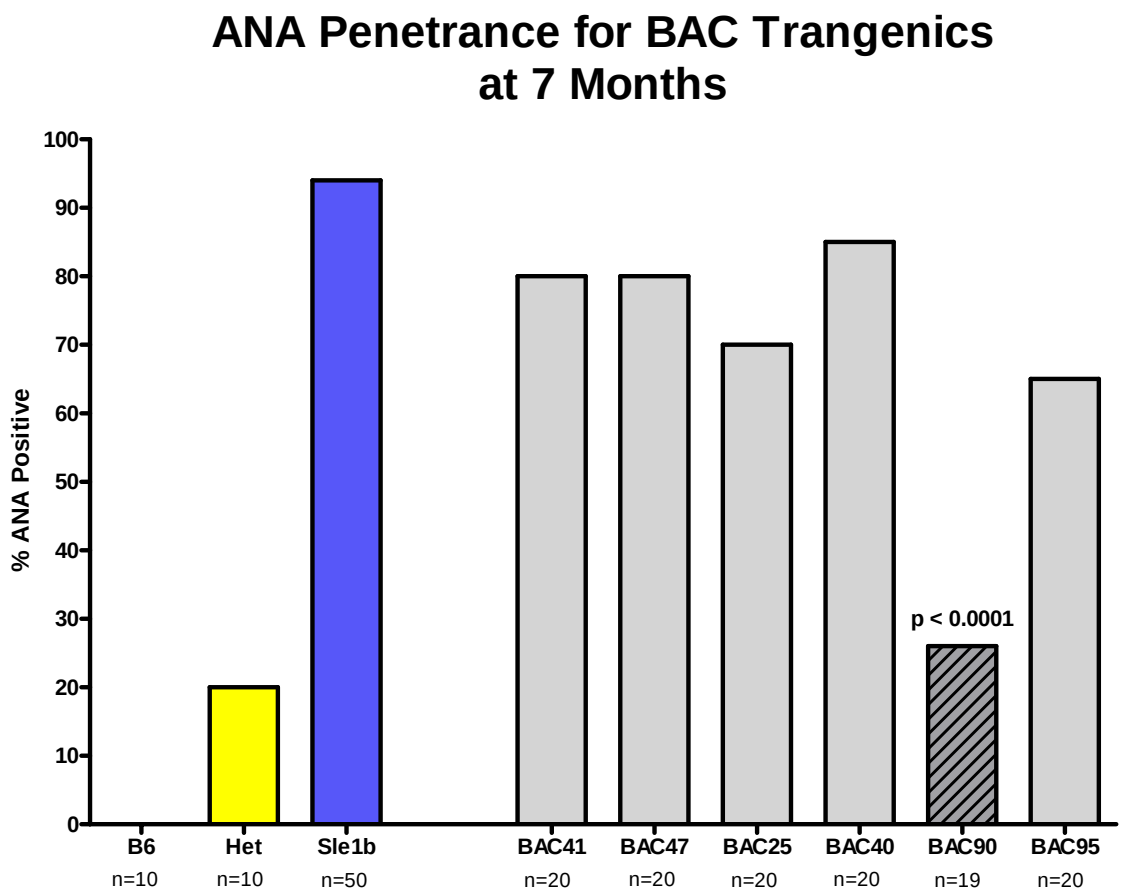


Figure 6. Only BAC90, containing Cc84 and Ly108, suppresses ANA production.

Six BACs carrying the B6 alleles of the *Sle1b* interval were crossed onto B6.*Sle1b* mice. Two founder lines for each BAC were assessed for IgG ANA at 7 months of age. Negative littermate controls for the founder lines were pooled to represent the ANA penetrance in B6.*Sle1b* mice. Only BAC90 suppressed ANA production that met statistically significance ($p<0.0001$). The remaining BACs had the following p values (BAC41 $p=0.28$; BAC47, $p=0.28$; BAC25, $p=0.87$, BAC40, $p=0.16$, BAC95, $p=0.49$). Statistical analysis was determined using logistic regression (SAS version 9.1).

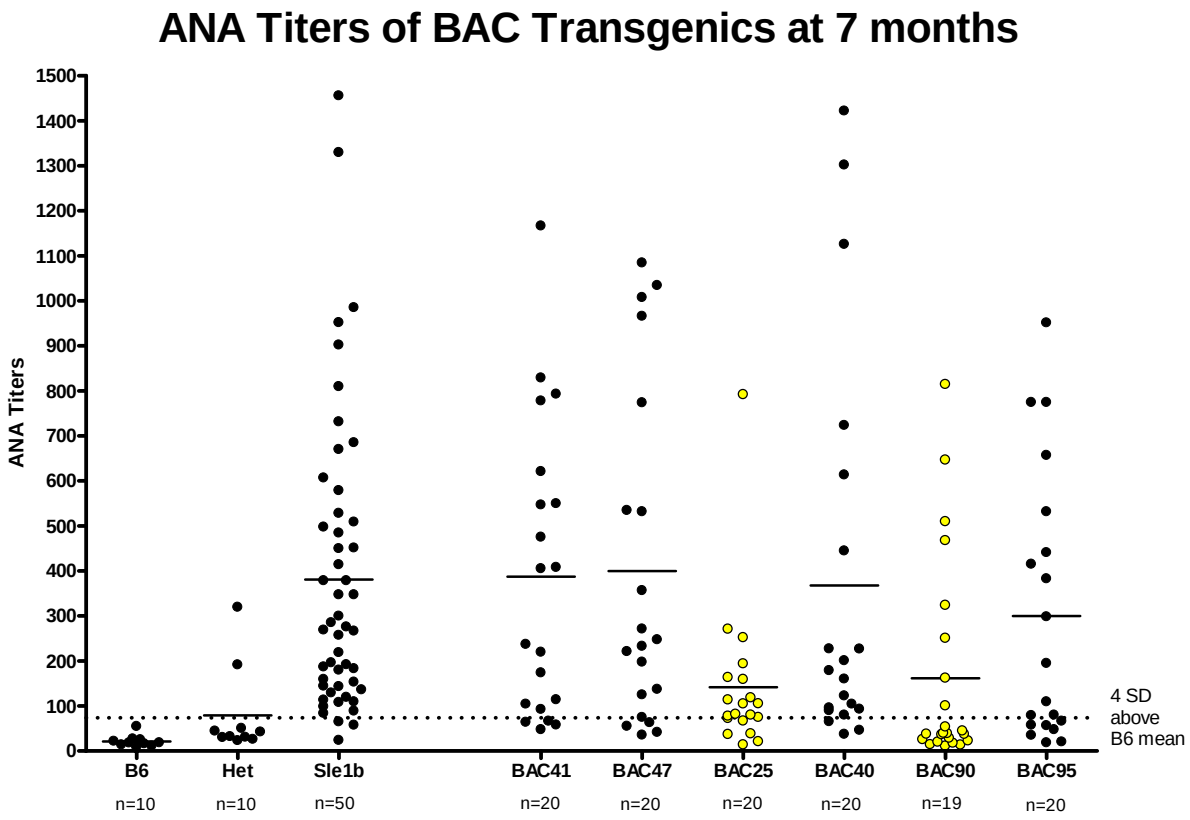


Figure 7. BAC25 and BAC90 have lower ANA titers than B6.Sle1b.

IgG ANA titers were assessed in 7 month old BAC transgenic mice. Dotted line represents the value that is four standard deviations above the mean titer from B6 mice. Solid line represents mean titer of each group. BAC25 (*2b4*, *Ly9* and *Cs1*) and BAC90 (*Cd84* and *Ly108*) are statistically significant from Sle1b ($p<0.01$ and $p<0.001$, respectively) using a non-parametric ANOVA (Kruskal-Wallis Test).

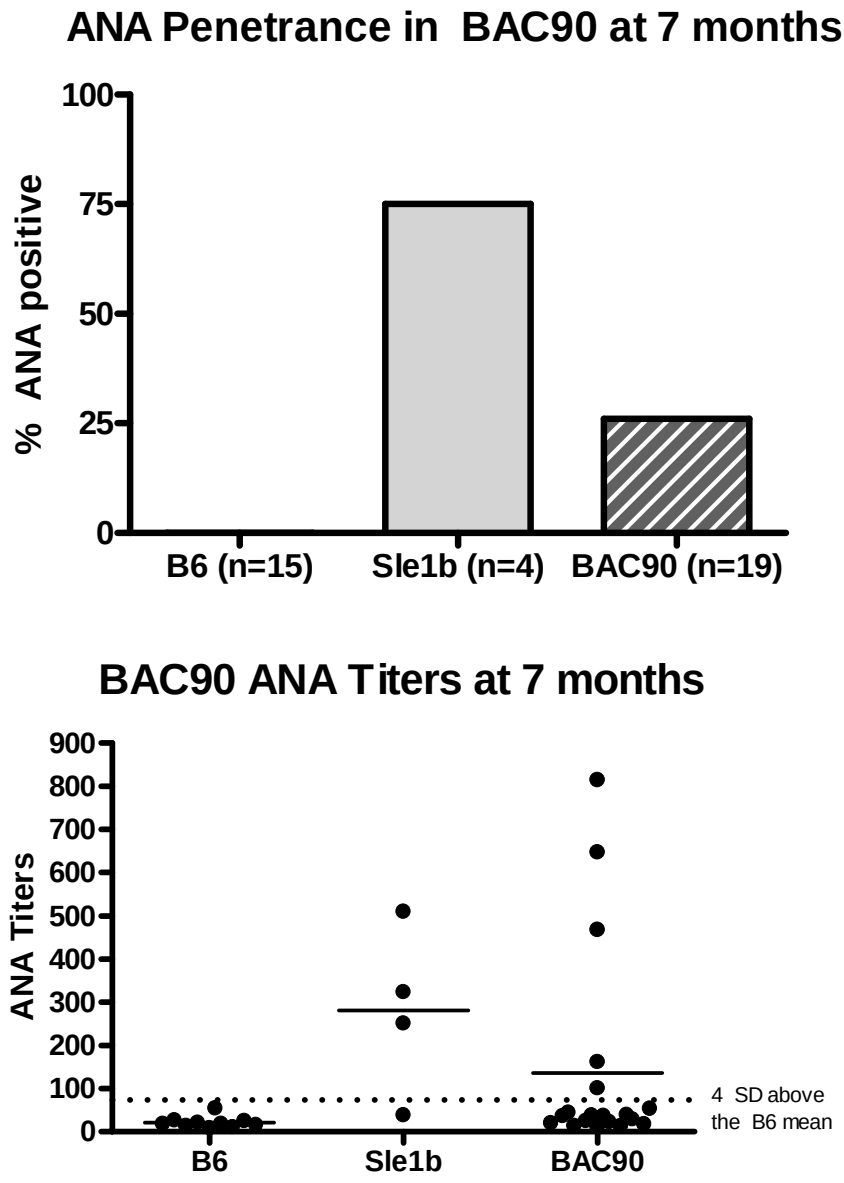


Figure 8. BAC90 suppresses ANA production on the B6.Sle1b background at 7 months

Sera were collected from 7 month old B6, B6.*Sle1b* (negative control littermates), and BAC 90 Tg mice (2 founder lines) and assessed for IgG ANA production. Top panel shows ANA penetrance and the bottom panel shows the titers of each individual mouse. Mice were considered ANA positive mice if the titers were four SD above the B6 mean.

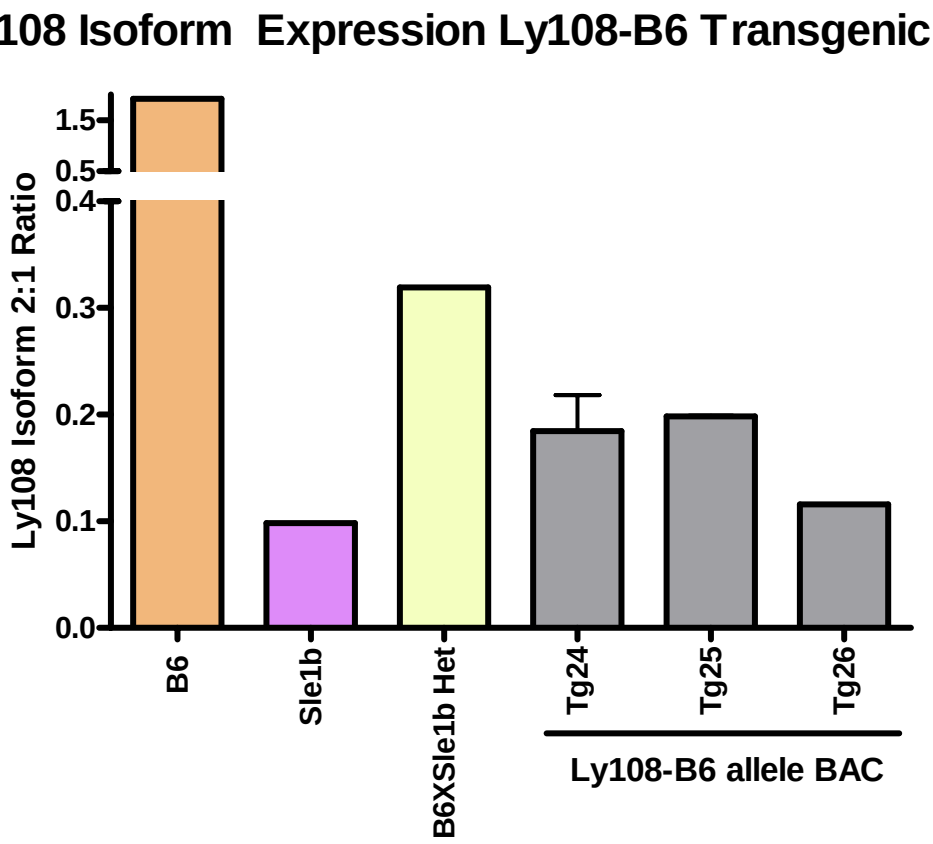


Figure 9. Increase in Ly108 isoform ratio in the B6 allele of *Ly108* BAC transgenic lines.

The Ly108 2:1 isoform ratio was assessed in BAC transgenic lines carrying the B6 allele of *Ly108* on the B6.*Sle1b* background. cDNA was synthesized from splenocytes, and isoform expression was assessed by quantitative real-time PCR. All three lines had increased ratios above B6.*Sle1b*, but not, however, to the level seen in a *Sle1b* heterozygote.

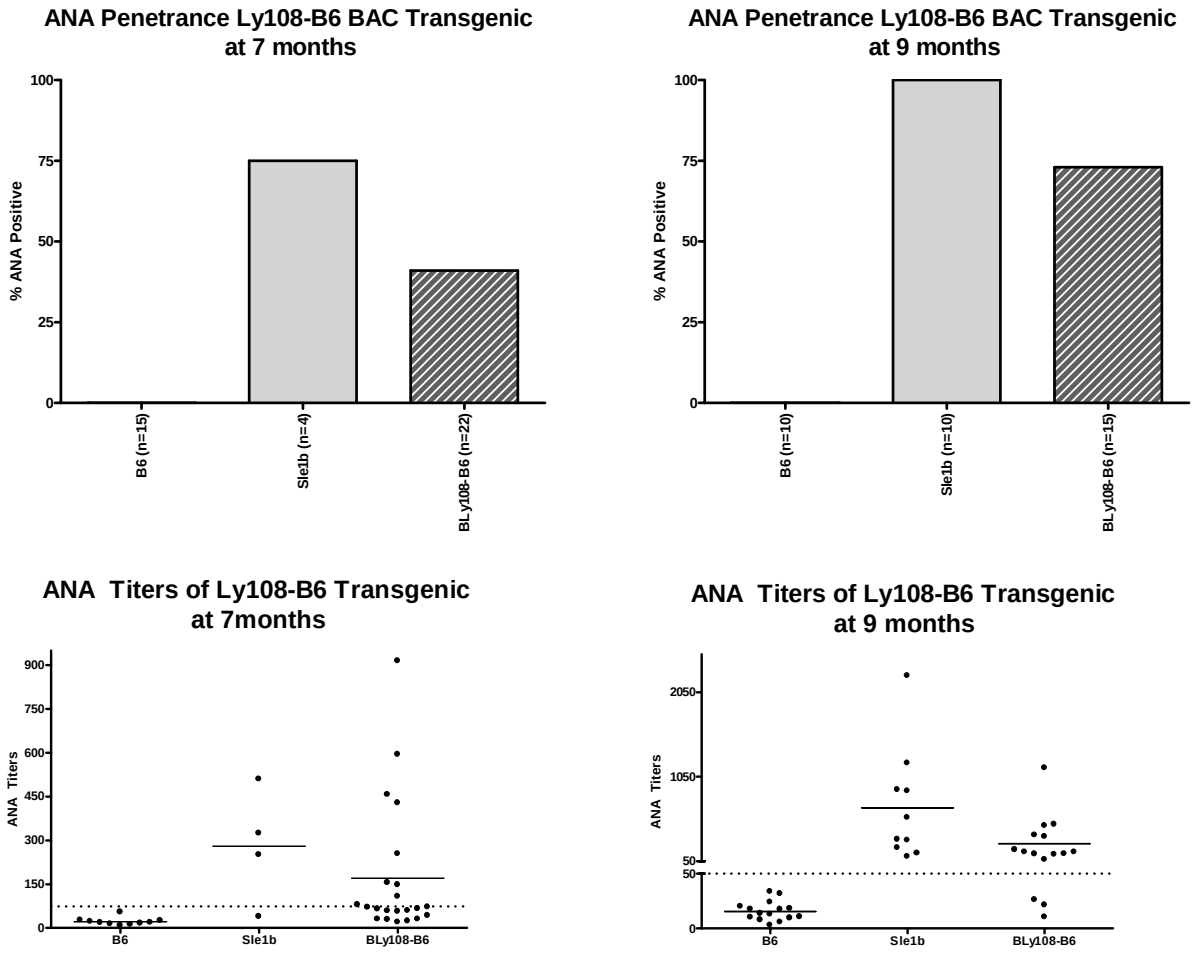


Figure 10. B6.*Ly108*-B6 BAC Transgenic mice have delayed kinetics in ANA production.

Sera were collected from B6, B6.*Sle1b,* and B6.*Ly108*-B6 mice (3 founder lines) at 7 and 9 months. IgG ANA production was assayed by ELISA. The top panels show ANA penetrance and bottom panels depict ANA titers. Mice were considered positive if the individual titer was greater than four standard deviations above the B6 mean (dotted line).

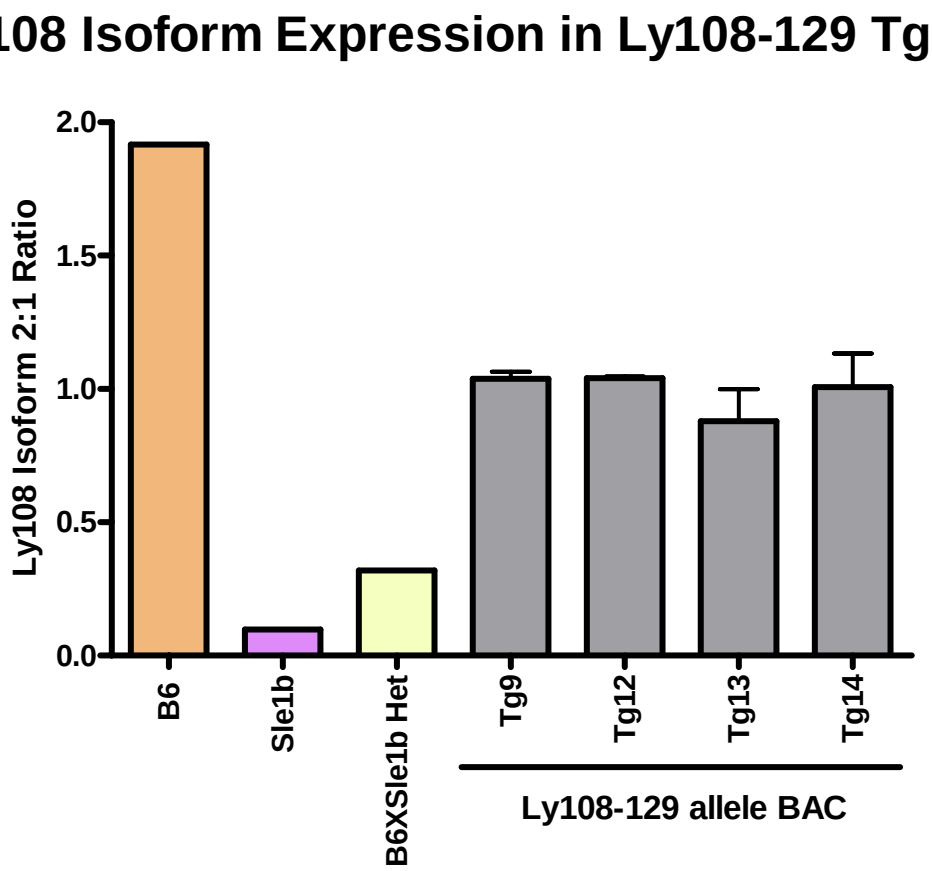


Figure 11. B6.*Ly108*-129 BAC transgenic lines have decreased *Ly108* isoform expression ratio.

The Ly108 2:1 isoform ratio was assessed in the different B6.*Ly108*-129 transgenic lines. cDNA was synthesized from splenocytes, and isoform expression was assessed by quantitative real-time PCR. All lines had an intermediate ratio between B6 and the B6.*Sle1b* heterozygote.

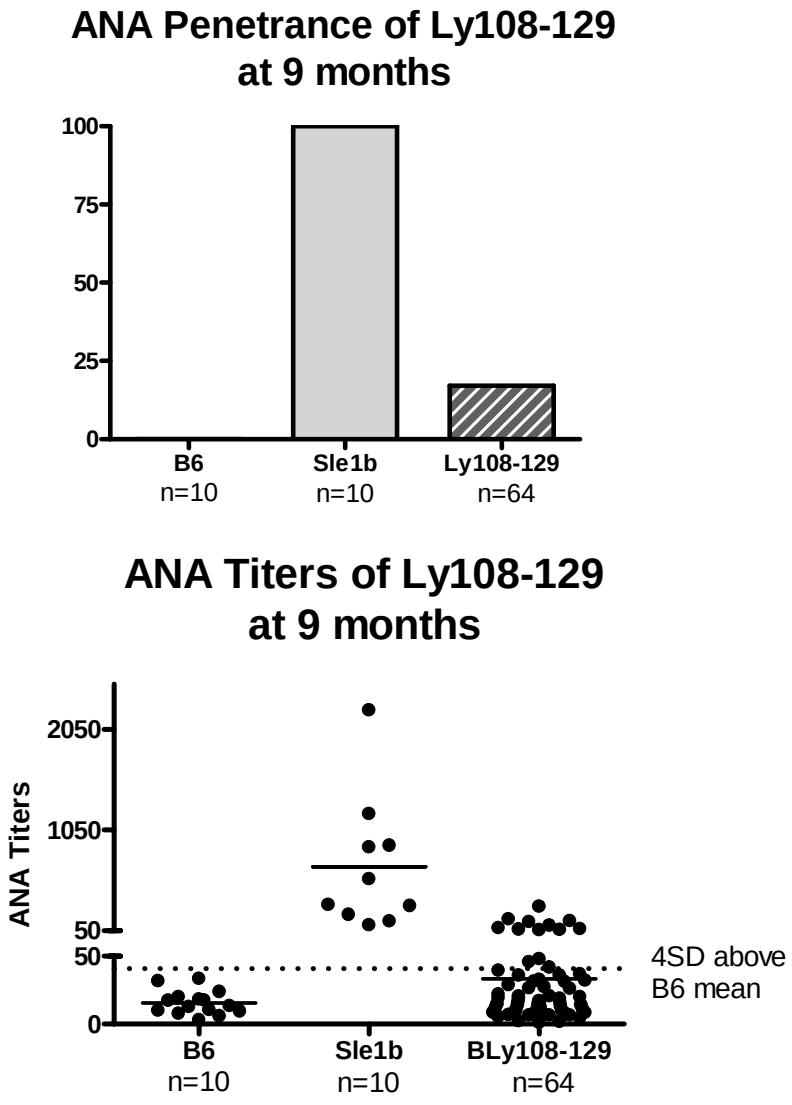


Figure 12. B6.*Ly108*-129 has increased ANA penetrance and titers.

Sera were collected from 9 month old B6 (negative control littermates), B6.*Sle1b*, and B6.*Ly108*-129 mice. IgG ANAs were assessed by ELISA and mice were considered positive if the individual titer was greater than four standard deviations above the B6 mean.

CHAPTER FIVE

Mechanistic Insight into SLAM family Modulation of Autoantibody Production Using Mouse Models

OVERVIEW

Numerous studies have demonstrated that the development of autoimmunity is accompanied by progressive dysregulation of a variety of immune cell subsets. It is believed that these phenotypes are reflective of intrinsic changes in immune cell populations that are a consequence of both genetic and environmental influences. B6.*Sle1b* mice are characterized by a loss in tolerance to chromatin and the production of IgG autoantibodies, but fail to develop fatal lupus nephritis. Despite this relatively mild form of humoral autoimmunity, with age both the lymphocyte and myeloid populations in these mice were shown to undergo significant alterations. These included increased activation and differentiation phenotypes, as well as changes in functional responses upon stimulation *in vitro*. However, because of the late age of onset of these phenotypes, it has been difficult to determine whether they represent intrinsic, genetically encoded modulations, or are simply a consequence of the ongoing autoimmune processes. Hence, identification of early changes in immune cell properties and functions that precede the development of autoimmunity in these mice could shed insight into underlying mechanisms.

Positional cloning analyses have implicated the SLAM family of immune receptors as the

key genes mediating the *Sle1b* phenotype determined by their by structural and expression differences (27). Functional characterization of B6.*Sle1b* mice identified early abnormalities in the CD4 T cell compartment that preceded the development of autoimmunity. The cells exhibited an altered calcium response and had an impaired ability to produce IL-4 upon stimulation (28). In the previous chapter, we identified *Ly108* as one of the causal genes for modulating autoantibody production *in vivo* using a series of BAC transgenic mouse models. By simply altering the isoform ratio of this gene *in vivo*, using BACs carrying allelic variants of *Ly108*, we were able to suppress and induce autoantibody production on the B6.*Sle1b* and B6 genetic backgrounds, respectively. However, how this gene functions to modulate tolerance and autoantibody production, and whether specific CD4 T cell and/or other immune cell abnormalities are necessary for the development of ANAs remained unknown.

Studies using mice with targeted disruptions in SLAM family genes, such as *Slam* and *Ly9*, have revealed that upon stimulation, the CD4 T cells have deficiencies in canonical T_H2 cytokine production, though at a lower magnitude than that seen in mice deficient in SAP, a downstream adaptor of the family. In order to gain insight into the mechanisms by which *Ly108* affects autoantibody production, we compared the responses of CD4 cells to stimulation *in vitro*. We wished to determine whether the *Sle1b* haplotype alone is sufficient to mediate changes in cytokine production, and how the differential isoform expression may influence these phenotypes. To address whether differences in cytokine production caused by haplotypic differences are necessary for autoantibody production, and to determine whether SAP is necessary for the autoimmune phenotypes, we introduced the SAP deficiency onto the

B6.*Sle1b* background.

RESULTS

The *Sle1b* Haplotype Modulates Cytokine Production Independent of Targeted Disruption of SLAM Family Genes

We recently generated a 129 congenic strain that carries the genomic segment containing the SLAM family from chromosome 1, which is approximately equivalent in size to *Sle1b*, on the B6 background. These mice (B6.*129Sle1b*) develop a loss in tolerance to chromatin similar in penetrance and kinetics to that seen in B6.*Sle1b* mice (Figure 13). When naïve CD4 T cells from young mice were purified by negative selection and stimulated with anti-CD3, with and without co-stimulatory stimulation through CD28, the cells exhibited an IL-4 defect as that described in the different SLAM family member knockouts, as well as in B6.*Sle1b* (Figure 14). Further characterization of the cytokine profiles post-stimulation *in vitro* revealed that the deficiency extended to other cytokines, including IL-5, IL-6, IL-10, and IL-13 (Figure 15).

These data demonstrate that, similar to autoantibody production, the haplotype mediates cytokine deficiencies in CD4 cells whether the genomic origin is from *Sle1b* or 129. Importantly, all the targeted disruptions of the SLAM family members (Ly9, SLAM, and Ly108) were generated in 129 ES cells, and the targeted regions, which carried the Sle1b/129 mutations, were then introgressed onto the B6 background. These knockout strains are essentially 129 congenics on B6 that lack specific SLAM family members. Given the

observed deficiencies in CD4 cytokine production in the 129 congenic line, in the absence of any targeted disruptions of SLAM family members, our data demonstrates that the haplotype by itself is sufficient to mediate these phenotypes. These results highlight the importance of using appropriate congenic controls when studying knockouts generated in 129 ES cells on the B6 background, similar to that seen in *Apcs*$^{-/-}$ mice (125).

The most pronounced expression difference in the autoimmune-promoting haplotype is the isoform utilization of *Ly108*. To explore whether specific *Ly108* isoforms can mediate the described IL-4 defect, we stimulated CD4 T cells from B6 and B6.*Sle1b* mice, which predominantly express the *Ly108-1* and *Ly108-2* isoforms respectively, using a monoclonal antibody to Ly108 that does not distinguish between the two of them. The addition of anti-Ly108, anti-CD3 and anti-CD28 antibodies to CD4 cells led to increased IL-4 production and a decrease in various inflammatory cytokines, such as TNFα, IFNγ and IL-6, as well as a decrease in IL-10 and IL-13 (Figure 16). However, there were no differences between the strains in terms of cytokine production following stimulation. Furthermore, engagement of Ly108 did not alter the isoform expression pattern of Ly108 in the strains (Figure 17). These data suggest that engaging Ly108, whether via Ly108-1 or Ly108-2, leads to increased IL-4 production.

Sle1b **Mediated Breach in Tolerance is Independent of SAP and IL-4 Deficiency**

SAP, encoded by *Sh2d1a* on chromosome X, is one of three known downstream adaptors of the SLAM family, which also includes EAT2 and ERT. Of these three known adaptors, SAP

is expressed in the widest variety of immune cell populations, including T cells, NK and NKT cells, and some B cells (79, 98, 99, 126). Given the known cellular expression requirements for *Sle1b* and the fact that the majority of SLAM family members are believed to signal via SAP, we wished to determine the effect of SAP deficiency on autoantibody production in B6.*Sle1b* mice.

B6.*Sle1b* mice were crossed to SAP knockout mice, thus generating B6.*Sle1bSh2d1a*$^{-/-}$ mice. Cohorts of females were aged and sera were collected at 7, 9, and 12 months to determine the requirements for SAP in autoantibody production in this model. As shown in Figure 19, despite the deficiency of SAP, the mice retained the ability to breach tolerance leading to the production of high titers of IgG autoantibodies. Hence, autoimmunity was able to develop in this model despite the many reported immune deficits present in SAP knockout mice, including the absence of NKT cells and long-lived memory B cells (99, 106, 107, 111). Furthermore, even when IL-4 production is abolished, as seen in the SAP knockout mice, autoantibody production was maintained. However, while the mice were able to breach tolerance, we did observe a reduction in the overall penetrance of autoantibody production when compared to B6.*Sle1b* mice of the same age (Figure 19). To ensure that absence of SAP did not alter the expression patterns of SLAM family members in B cells, we compared their expression in naïve B cells in the presence or absence of SAP. As depicted in Figure 20, we did not detect any differences in the expression of SLAM family members regardless of SAP expression.

Cumulatively, these data indicate that while SAP is not required for the production of the high titered IgG autoantibodies seen in B6.*Sle1b* mice, it appears that it is necessary for the augmentation of the response and full recapitulation of the penetrance of this breach in tolerance. Furthermore, it appears that decrease IL-4 secretion as seen in B6.*Sle1b* by itself does not cause the development of autoantibodies, as SAP knockout mice have a complete deficiency in IL-4 production and do not develop highly penetrant autoantibody production.

SUMMARY

In this chapter we presented evidence that subtle functional and structural polymorphisms seem in the *Sle1b* SLAM family haplotype modulate cytokine secretion in CD4 cells post-stimulation. Although we were able to identify a causative allele of *Ly108* for mediating the autoantibody production seen in B6.*Sle1b* mice, it has been difficult to determine mechanistically how this gene actually functions to cause the breach in tolerance. The data described in this chapter presents two key pieces of evidence that shed light on mechanistic requirements in the pathway leading to Ly108 mediated autoantibody development.

The SLAM family of immune receptors have been implicated in a variety of immune functions and have been postulated to serve as adhesion and co-stimulatory molecules, based on their presence on multiple cell types, starting at the earliest stages of hematopoietic stem cell development, and the nature of their homophilic and heterophilic interactions. Many of the attributed roles of these receptors have come from studies of knockouts of different family members. Key amongst these functions has been the involvement of the SLAM family

in modulation of cytokine secretion in CD4 cells, and a role in these processes has been implicated for SLAM, Ly9 and Ly108. We demonstrate that some of these phenotypes are a consequence of introgressing the *Sle1b* SLAM family haplotype present in 129 onto the B6 background during the generation of the knockout strains. We found that both the *Sle1b* and 129 SLAM family congenics show decreased cytokine production, in particular IL-4, post-stimulation of CD4 cells *in vitro*. These data demonstrate that this phenotype was an intrinsic property of T cells bearing this haplotype and not a consequence of the absence of any particular SLAM family member.

We next examined the consequences of Ly108 stimulation, and established that differential engagement of the two isoforms of Ly108 did not alter the cytokine profile between B6 and B6.*Sle1b*. Engagement of either isoform in purified CD4 T cells was able to increase IL-4 production and decrease secretion of other cytokines, such as TNFα and IFNγ. Whether this is due to the antibody acting in a blocking or activating manner remains to be determined.

Finally, we ascertained that the breach in tolerance is not caused by IL-4 deficiency, and that Ly108 is mediating autoantibody production in a SAP-independent manner. These data are unique in that to date, all reports have revealed that SAP is required for the development of lupus in different model systems. However, our data does indicate that SAP plays a critical role in enhancing the penetrance of autoantibody production. This was not due to changes in the expression pattern of SLAM family members in B cells in the absence of SAP. These data suggest that the mechanism(s) by which Ly108 mediates breach/maintenance of

tolerance is not critically dependent on SAP expression. Given these data, and the known diversity in cellular expression patterns of the SLAM family, which is not observed for any of the known adaptor molecules, it implies the existence of a currently unknown SLAM family adaptor that plays a pivotal role in the signaling processes mediated by Ly108 and its ability to modulate tolerogenic mechanisms.

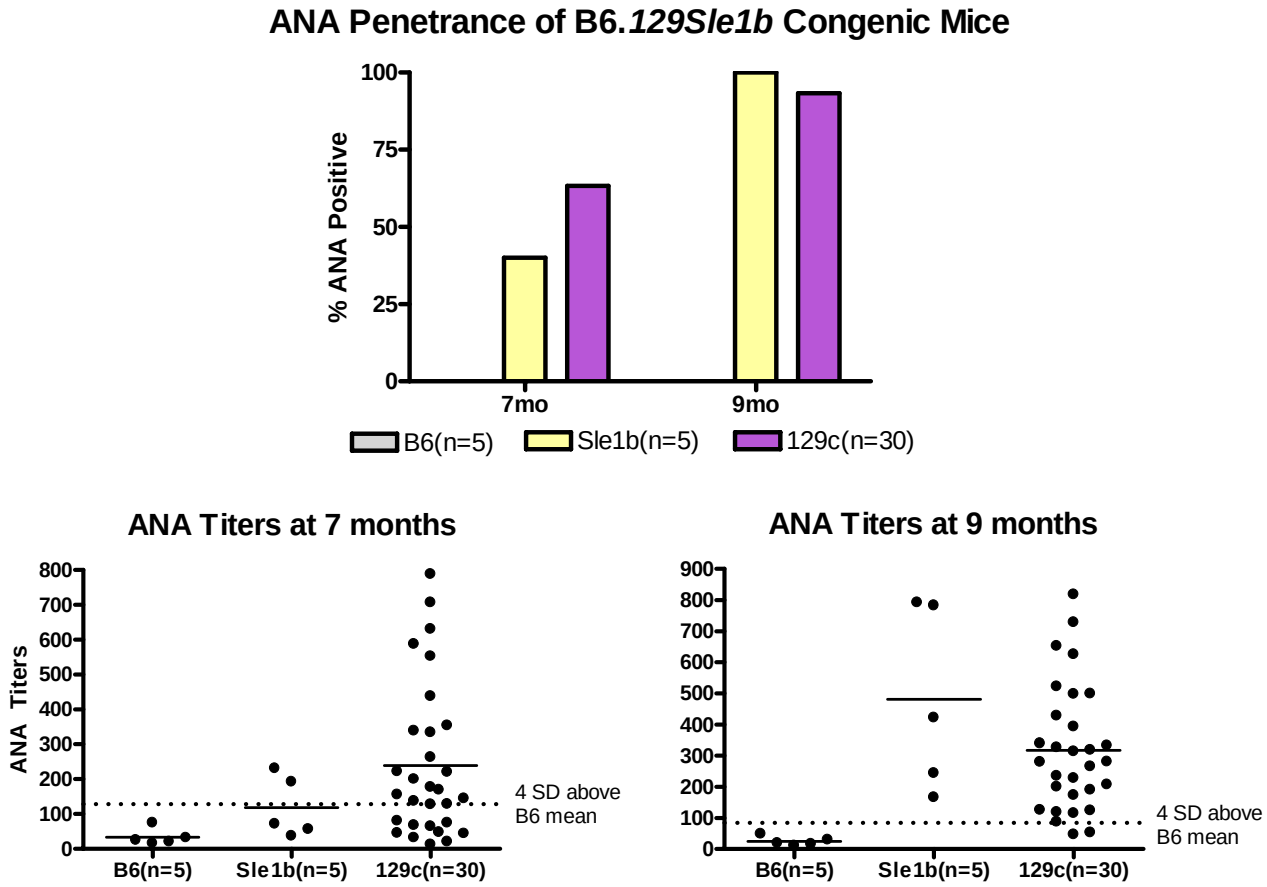


Figure 13. Penetrance and titers of autoantibodies in B6.*129Sle1b* congenic mice are similar to B6.*Sle1b*

B6, B6.*Sle1b*, and B6.*129Sle1b* (both congenic lines) congenic mice were aged and sera collected at 7 and 9 months. IgG anti-chromatin production was measured by ELISA. Top panel depicts penetrance and the bottom two panels show titers. Mice were considered positive if the titers were greater than four standard deviations above mean of the age-matched B6 controls (dotted line).

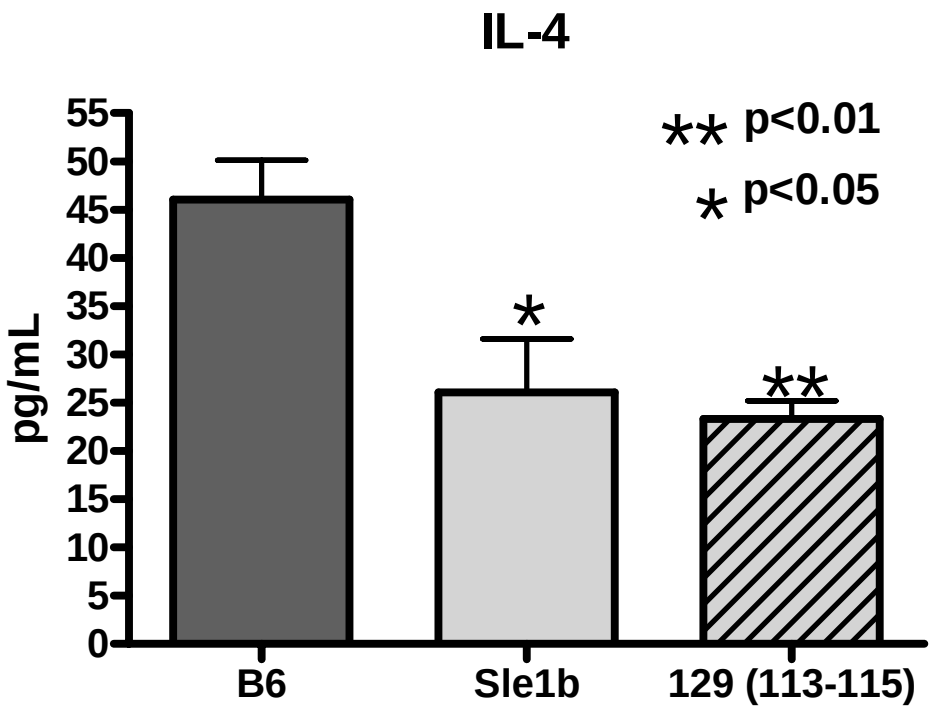


Figure 14. Decreased IL-4 production in both B6.*Sle1b* and B6.*129Sle1b* congenic mice

Naïve CD4 T cells from 6 week old mice were stimulated with plate-bound anti-CD3 (1ug/mL) plus anti-CD28 (10ug/mL) for 72 hours. Supernatants were collected and assessed for IL-4 production by Cytokine Bead Array. Both B6.*Sle1b* and 129 congenic mice showed a statistically significant reduction in IL-4 production in comparison to B6 (one-way ANOVA, Dunnett's Multiple Comparison Test) (n=4 per genotype).

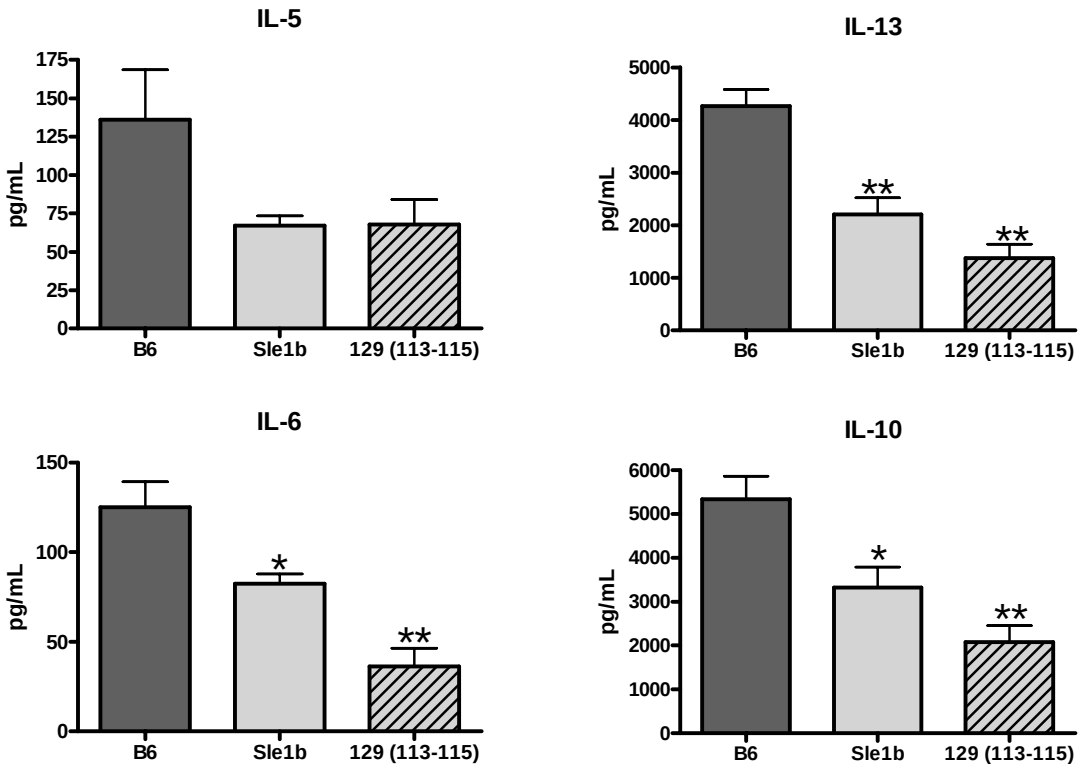


Figure 15. B6.*Sle1b* and B6.*129Sle1b* congenic mice have reduced IL-5, IL-13, IL-6, and IL-10 production.

Naïve CD4 T cells from 6 week old mice of the indicated genotypes were stimulated with plate-bound anti-CD3 (1ug/mL) plus anti-CD28 (10ug/mL) for 72 hours. Supernatants were collected and assessed for cytokine production by Cytokine Bead Array. B6.*Sle1b* and 129 congenic mice showed a reduction in IL-5, IL-13, IL-6, and IL-10 production compared to B6. (*$p<0.05$ and **$p<0.01$ measured by one-way ANOVA followed by a Dunnett's Multiple Comparison Test) (n=4 per genotype).

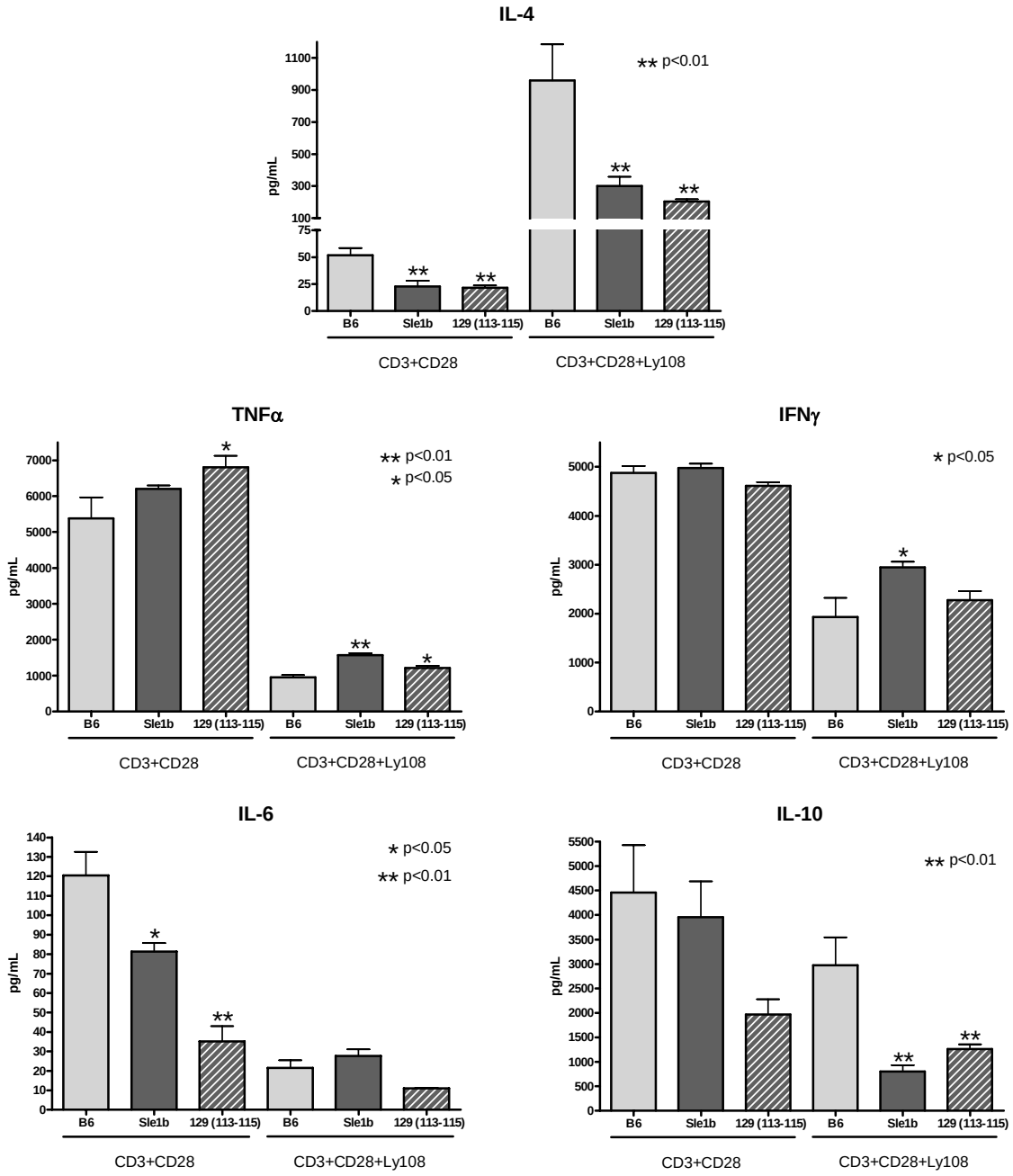


Figure 16. Modulations in CD4 T cell cytokine production with anti-Ly108 plus anti-CD3 and anti-CD28 stimulation.

Naïve CD4 T cells purified from 6 week old mice of the indicated genotypes were stimulated with anti-CD3, anti-CD28, and anti-Ly108 for 72 hours. Supernatants were assessed for

cytokine production by CBA. One-way ANOVA followed by a Dunnett's Multiple Comparison Test with B6 in each stimulation condition as the control was performed. (*$p<0.05$ and **$p<0.01$) (n=5 per genotype)

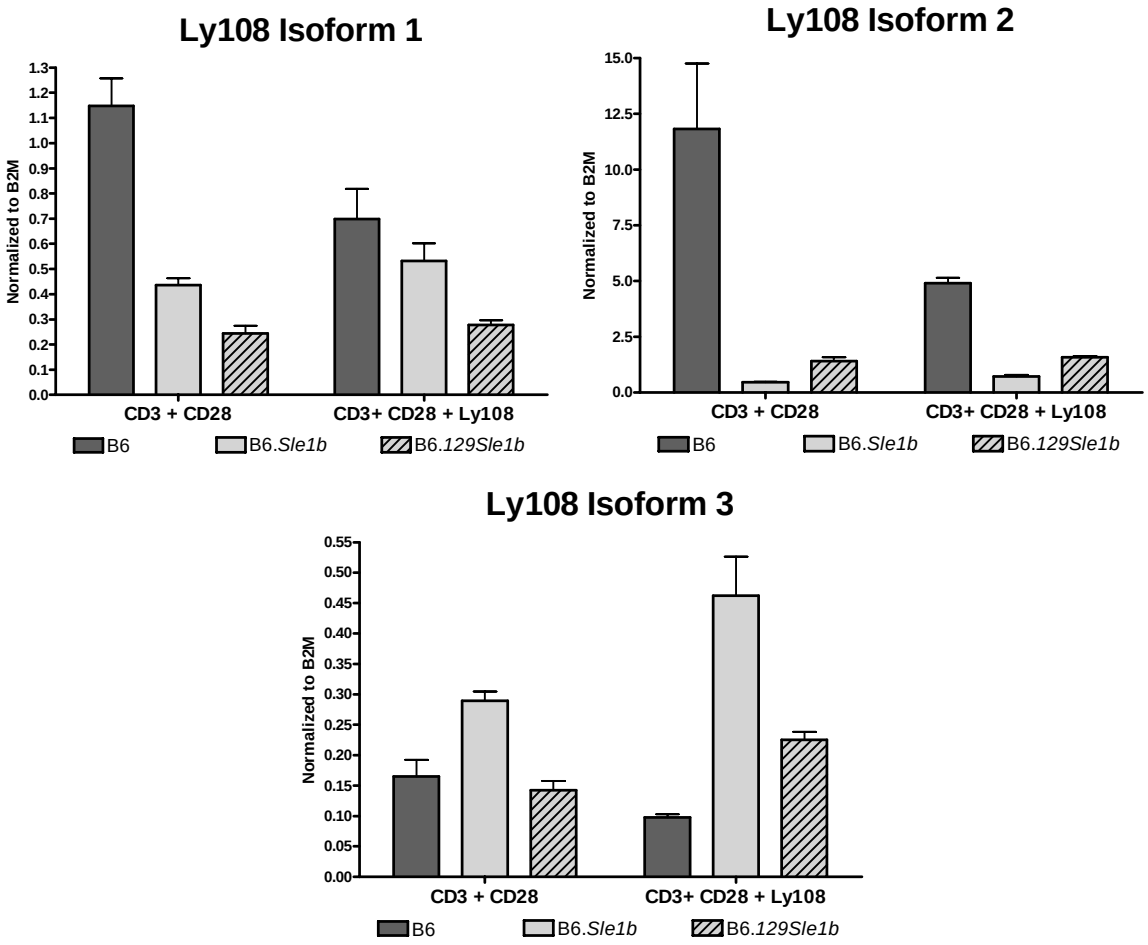


Figure 17. Ly108 isoform utilization is maintained with the addition of anti-Ly108.

Naïve CD4 T cells were stimulated with anti-CD3 and anti-CD28 with or without anti-Ly108 for 24 hours. Cells were harvested for RNA and Ly108 isoform expression was assessed by real-time PCR. Anti-Ly108 does not alter the isoform utilization pattern between the different strains. (n=5 per genotype)

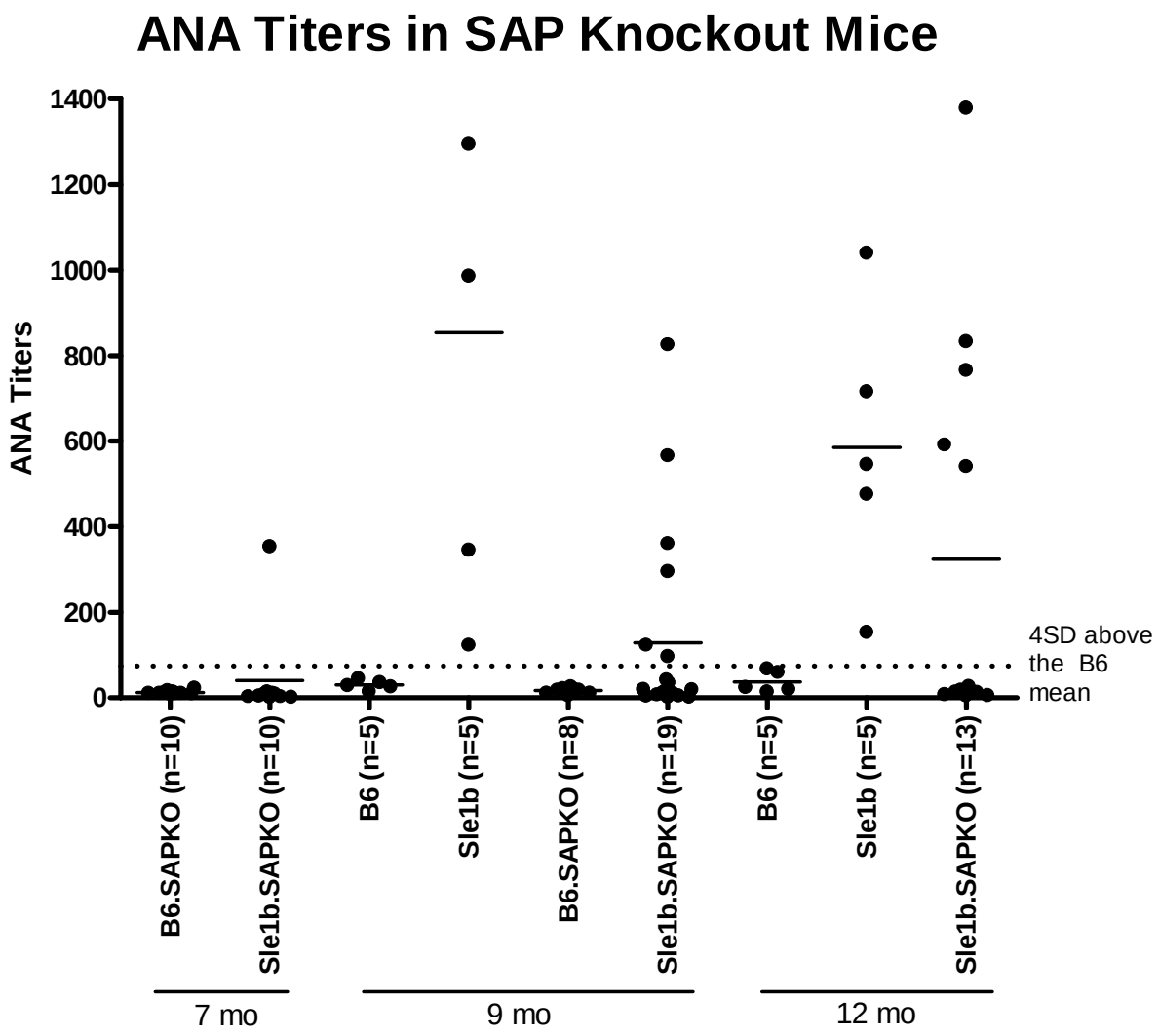


Figure 18. Breach in tolerance mediated by *Sle1b* is maintained despite the lack of SAP function.

Female mice of each genotype were aged and sera collected at 7, 9, and 12 months of age. IgG ANA titers were assessed by ELISA. Dotted line represents four standard deviations above the B6 mean titer. *Sle1b* on SAP knockout breached tolerance to nuclear antigens whereas B6.SAPKO mice did not.

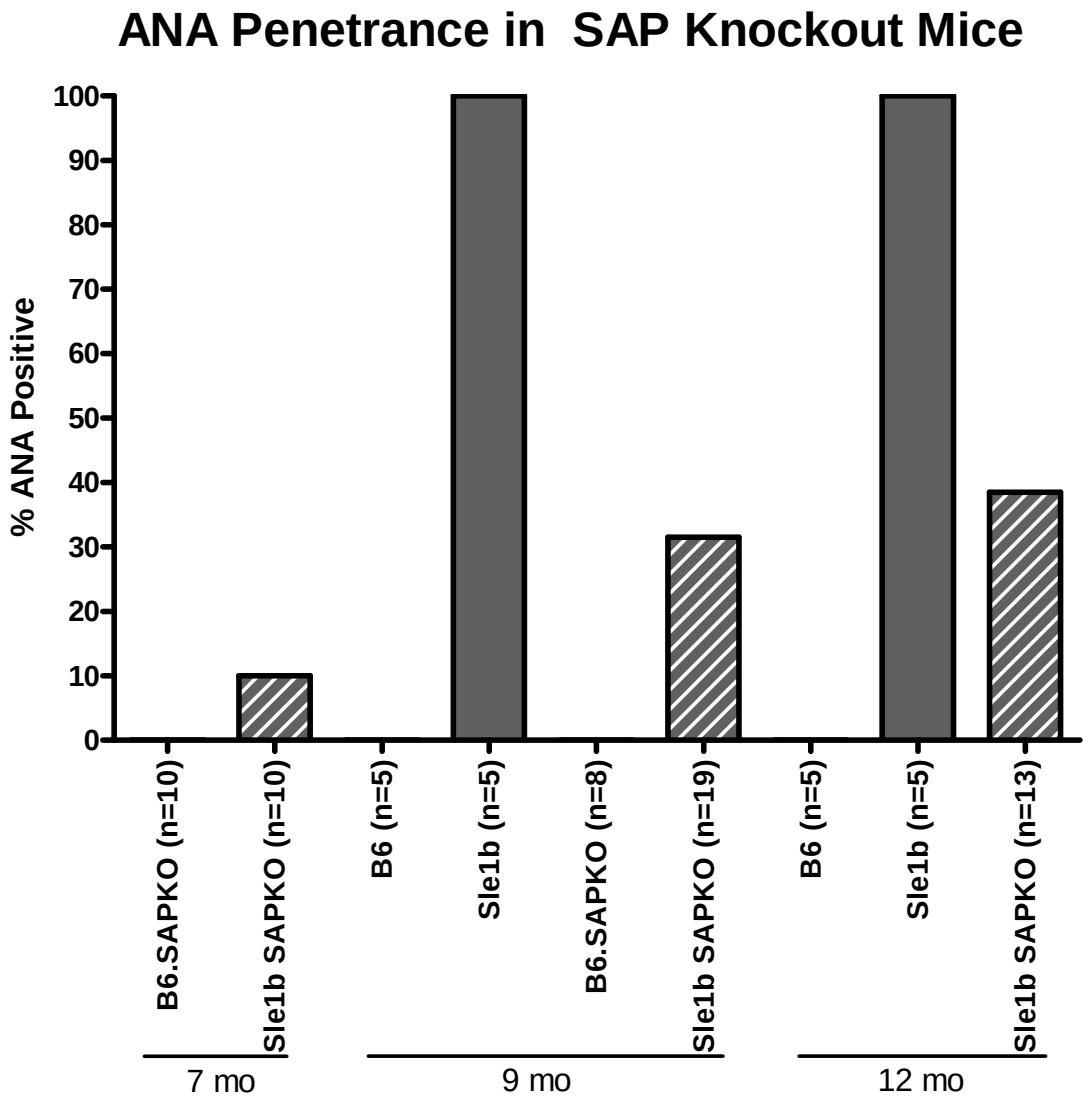


Figure 19. ANA penetrance is reduced in the absence of SAP.

Female mice of the indicated genotypes were aged and sera collected at 7, 9 and 12 months. IgG anti-chromatin production was measured by ELISA. Mice were considered positive if the titers were greater than four standard deviations above the mean of the age-matched B6 controls. In the absence of SAP, *Sle1b* mediated autoantibody production is maintained, though at reduced penetrance.

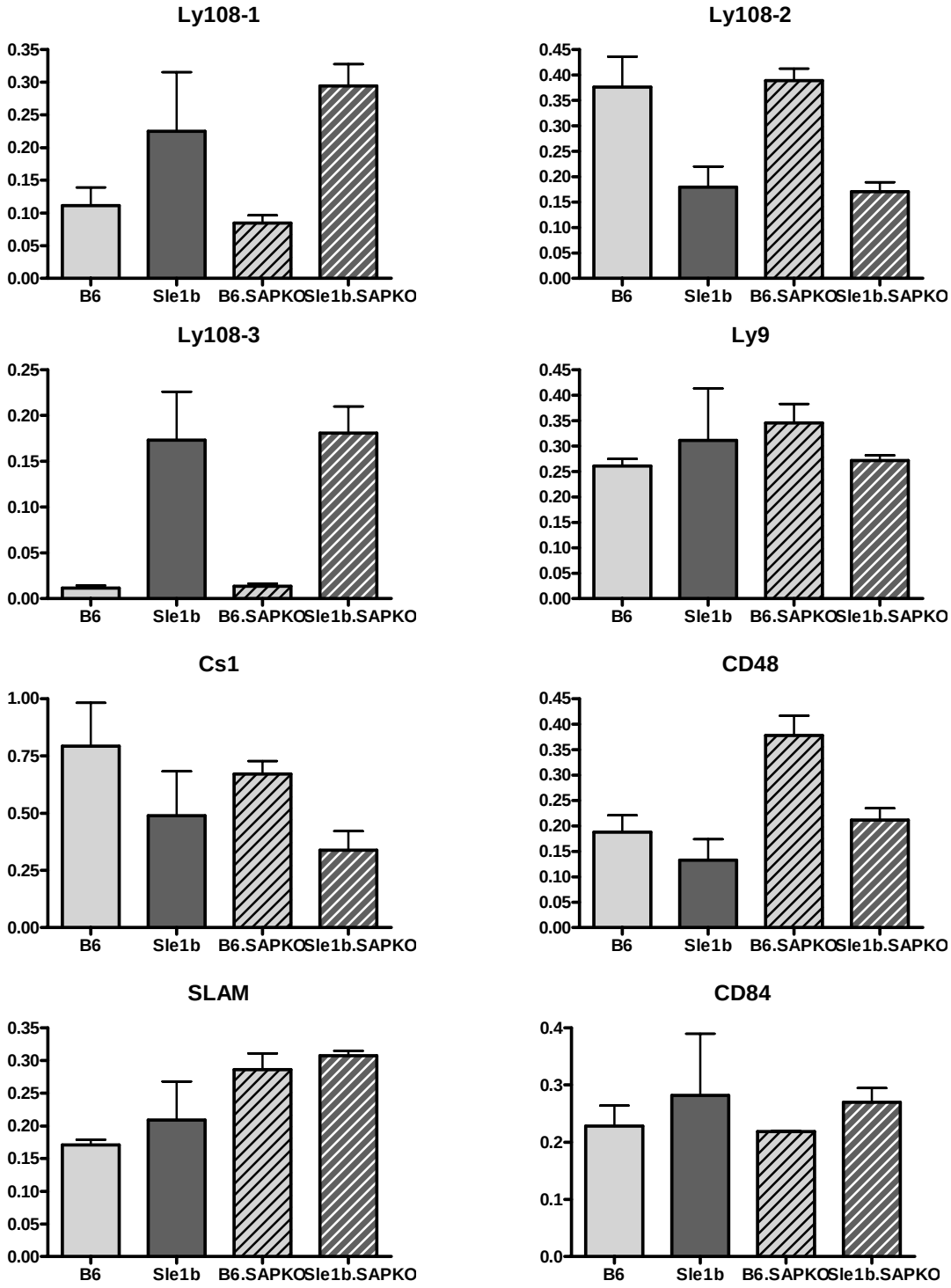


Figure 20. SLAM family expression in naive B cells.

The expression pattern of SLAM family members was assayed using real-time PCR in naïve

B cells isolated from B6, B6.*Sle1b*, B6.SAPKO, and B6.*Sle1b*.*SAPKO* mice (n=3 per genotype). SAP deficiency does not alter the expression pattern between B6 and B6.*Sle1b* mice.

CHAPTER SIX

Discussion

CAUSATIVE GENE IDENTIFCATION IN A COMPLEX GENETIC DISEASE

Complex genetic diseases, like SLE, arise from the effects of specific combinations of alleles within multiple susceptibility loci that interact in various pathways to progress towards disease onset. The majority, if not all such diseases, are believed to be attributable to slight alterations in gene expression or structural protein modifications, rather than the lack of a functional gene. It is the subtlety of these changes that add further complexity to the identification and characterization of causative alleles in polygenic diseases.

In the NZM2410 model of murine lupus, we have identified four disease susceptibility loci (20). When all loci were reassembled on to the B6 lupus-resistant background, we were able to fully recapitulate the disease phenotype seen in NZM2410 (22). To dissect the component phenotypes that each individual locus was mediating, a congenic dissection approach was used, in which one given disease susceptibility locus was introgressed onto the B6 background. The locus on the telomeric end of chromosome 1, termed *Sle1*, mediated a breach in tolerance to nuclear antigens (23). Furthermore, it was shown that the initial loss in tolerance mediated by *Sle1* is necessary for the generation of the full spectrum of clinical phenotypes when additional susceptibility loci were present (22). Fine mapping analyses demonstrated that *Sle1* was actually composed of four sub-loci, *Sle1a-d*. *Sle1b* was the most potent of all the sub-loci and had the capacity to recapitulate by itself the autoantibody

production phenotype seen in B6.*Sle1* (26). To identify the gene responsible for the *Sle1b* phenotype, genetic and expression polymorphisms were assessed, and the most dramatic changes were in those genes belonging to the SLAM/CD2 family (27). Seven family members, *2B4*, *Ly9*, *Cs1*, *Cd48*, *Slam*, *Cd84*, *and Ly108*, are contained within the 940 Kb *Sle1b* interval, and sequence polymorphisms distinguished two stable haplotypes, with the *Sle1b* haplotype being the more common of the two in the panel of inbred mouse strains examined (29). By introgressing the *Sle1b* haplotype from either 129 or Cast/Ei onto the B6 genome, ANA production similar to *Sle1b* was recapitulated (27). These data demonstrated that the SLAM/CD2 alleles are not unique to *Sle1b* and 'autoimmune-prone' strains in general, but rather are the common variants in the population. This suggests that these alleles are being maintained in natural populations, quite possibly by pathogen-driven selection mechanisms, and provide evidence to support the hypothesis that specific and unfortunate combinations of common alleles are the underlying basis for complex diseases.

GENE(S) MEDIATING ANA PRODUCTION IN B6.*SLE1B*

2b4 Gene Expansion is Not Likely Mediating the *Sle1b* Phenotype

The *2b4* gene in the *Sle1b* haplotype has undergone a gene duplication event, such that four copies of the gene are present, instead of the single copy seen in the B6 haplotype. Of the four types, at least one, type 3, is a pseudogene, due to an early stop codon present in the extracellular domains. Type 4, the most divergent type from B6, was readily detectable in cDNA from splenocytes and LAK cells from the *Sle1b* haplotype. The degree of differences

present between the most prevalent types in the two haplotypes explains the allelic differences that have been previously reported (121, 122). When engaged by an antibody, these two types also have opposing functional responses. The B6 allele leads to an inhibition of NK cytotoxicity, whereas the *Sle1b 2b4* allele increases the cytotoxic response (127). The functional consequences of engaging the remaining two 2B4 types are currently unknown. Although type 1 has the most resemblance to B6 and could potentially function like its B6 counterpart, its expression is low in splenocytes, similar to type 2. It is possible that these two types may have evolved to be expressed in a small subset of immune cells. It is worth noting that the analysis of the differences between all the types was focused on the extracellular domain. We were unsuccessful at identifying the cytoplasmic domains using primers designed from the B6 sequence suggesting that the cytoplasmic domains in these duplicated genes, if present, may have mutated extensively. These domains could potentially function in a completely novel manner and recruit alternative and/or currently unknown adaptors. Studies have been initiated to investigate the different functional roles of the two most prevalent 2B4 types.

We also extended our analyses of the *2b4* expansion to wild-derived mice, which showed greater complexity in their pattern of expansion. This may simply reflect greater diversification of the *2b4* gene expansion as a result of pathogen-driven selection mechanisms. While there is no data implicating 2B4 in pathogen responses, two members of the SLAM family, CD48 and SLAM, have been shown to be receptors for the pathogens *E. coli* and measles virus, respectively (69, 74). Moreover, it was shown that the loss of Ly108

or SLAM functions led to increased susceptibility to bacterial infections, while SAP has been shown to play a critical role in modulating the immune response during EBV infection (84, 85, 105, 109). It is hence tempting to speculate that the expansion of *2b4*, and the evolution of this family in general, is a consequence of pathogen-driven selection mechanisms, due to the ability of this family to serve as receptors for pathogens and modulate the immune response in order to appropriately control infectious agents.

Among the wild mouse strains analyzed, Cast/Ei was particularly noteworthy. We showed that the Cast/Ei haplotype was able to mediate ANA production when introgressed onto the B6 background. Interestingly, this strain possesses a single copy of the *2b4* gene, indicating that the SLAM family haplotype in Cast/Ei is actually a recombinant between the B6 and *Sle1b* haplotypes, such that the proximal end is like B6, while the distal end is similar to that of *Sle1b*. Sequence analysis of the extracellular domain of *2b4* from Cast/Ei confirmed that the single *2b4* gene present in Cast/Ei shares more similarity to B6 than the most prevalent form in 129 and the *Sle1b* haplotype. Given the ability of the Cast/Ei haplotype to mediate autoantibody production, these data indicate that *2b4* is not the causal gene.

Ly108 is Sufficient for Mediating the Loss of Tolerance to Nuclear Antigens

To narrow the search for the causative allele in the ~940 Kb *Sle1b* interval, we utilized a BAC genetic complementation approach, whereby a series of BAC transgenic mice carrying B6-alleles that spanned the *Sle1b* interval were generated and crossed to B6.*Sle1b* mice. Based on the known allele-dose dependent effect of the *Sle1b* gene, only the BAC harboring

the causative allele would be predicted to suppress autoantibody production. Of the six BACs in the tiling path, only BAC90, expressing the B6 alleles of *Cd84* and *Ly108*, mediated a dramatic suppression of the *Sle1b* phenotype.

One of the two genes present on BAC90, *Ly108*, exhibited a pronounced difference in isoform utilization between the two haplotypes (B6 and *Sle1b*), and thus was our most promising single gene candidate. To determine whether *Ly108* was the causative gene, we generated two BAC transgenic models. One carried the lupus-resistant (B6) allele of *Ly108* on the B6.*Sle1b* genetic background, and the other harbored the lupus-susceptible (129) allele of *Ly108* on the lupus-resistant B6 background. The presence of the transgene carrying the B6 allele on the *Sle1b* genetic background was able to shift the isoform ratio towards that of B6. Conversely, expression of the BAC harboring the 129 allele on the B6 background changed the ratio in the opposite direction towards that of *Sle1b*. These subtle shifts in Ly108 isoform ratio were able to significantly alter the autoantibody phenotypes of their respective backgrounds. The B6-allele of *Ly108* suppressed ANA production on the lupus-susceptible B6.*Sle1b* background, while the 129 allele was able to mediate a breach in tolerance. Taken together these data clearly identify *Ly108* as playing a role in ANA development and pathogenesis.

Although we have identified *Ly108* as a causative gene for mediating the loss in tolerance, how it functions in this capacity remains to be determined. Phenotypes from the knockout of *Ly108* revealed a role for this molecule in regulating cytokine production in both CD4 T cells

and myeloid cells (95, 97). Ly108 may modulate the cytokine milieu in a given niche to promote activation and/or survival of autoreactive cells. *In vitro* stimulation using an anti-Ly108 antibody demonstrated that it could promote IL-4 production and inhibit inflammatory cytokine production. This was independent of the genotype of the mouse where B6 expresses predominantly Ly108-2 and B6.*Sle1b* expresses mainly Ly108-1. Since the difference in IL-4 production between B6 and B6.*Sle1b* was present irregardless of Ly108 stimulation, it is feasible to speculated two possibilities. First, the signaling capacity for mediating IL-4 production between the two Ly108 isoforms is equivalent. And second, the bias in Ly108 isoform usage likely does not account for the intrinsic IL-4 defect upon stimulation. However, for the inflammatory cytokines B6 and B6.*Sle1b* did respond differently when stimulated with Ly108 antibody. It is possible that the different Ly108 isoforms may potentially be mediating these intrinsic cytokine differences. For example, IL-10 production was significantly reduced in B6.*Sle1b* and B6.129*Sle1b* congenics when the TCR and Ly108 were co-engaged. Based on the known roles of IL-10, we can speculate that the differential ability of *Ly108* isoforms in mediating decreased IL-10 production may help promote T cell activation and, via SAP, memory B cell development (128). Ly108 isoforms also have the potential to impact apoptosis and BCR signaling (K.R. Kumar, *Submitted*). It is possible that the differential expression of levels of the two isoforms can breach normal tolerance mechanisms by altering the BCR signaling threshold or by promoting the survival of autoreactive cells.

It has been shown that the ITSMs in *Ly108* bind SAP, a downstream adaptor of several

members of the SLAM family (91, 93). However, we have shown that Ly108 signaling via SAP is not necessary for mediating the loss in tolerance. The other known adaptors for this family, Eat-2 and ERT, are not expressed in T cells or B cells, suggesting the possibility of an unidentified adaptor. To identify potential adaptors, we have performed microarray analyses on purified splenic B and CD4 T cells to examine differences in SH2-containing genes between B6 and B6.*Sle1b* mice. However, it is possible that this adaptor may not be differentially regulated at the transcriptional level.

Other SLAM Family Members Potentiate Ly108 Function to Mediate the *Sle1b* Phenotype

BAC25 genes 2b4, Ly9, and/or Cs1

Based on the data from the BAC transgenic models, there are two components of the *Sle1b* phenotype. The first component is the actual breach in tolerance, while the second involves the development of fully penetrant, high titer ANA production. The loss of tolerance in the Ly108-129 transgenic demonstrates that *Ly108* is a causal gene sufficient to mediate this phenotype. However, these mice only produced low titer ANA, suggesting that another gene within the *Sle1b* interval is needed to recapitulate the full phenotype. BAC25, which carries the B6 alleles of *2b4*, *Ly9* and *Cs1*, on the B6.*Sle1b* background inhibited the amount of ANA generated, but failed to suppress the number of mice breaching tolerance, *i.e.* penetrance. This illustrates that an allele within BAC25 may be potentiating the breach in tolerance initially mediated by *Ly108*. Thus, we anticipate that reconstituting a B6 mouse with the *Sle1b*/129 alleles of *Ly108* plus *2b4*, *Ly9*, and/or *Cs1* would lead to fully penetrant,

high titer ANA production and recapitulate the B6.*Sle1b* phenotype.

SAP

Several groups have also studied the role of SAP in other murine models of lupus. In a MRL/*lpr* model carrying a spontaneous mutation of SAP, the mutant clearly exhibited high titer ANA relative to MRL/+, but lower IgG ANA titers in comparison to MRL/*lpr* (112). Consistent with this report, our data suggests that SAP acts synergistically with the *Sle1b*/129 allele of *Ly108* to enhance the production of autoantibody, although it is not necessary for breaching tolerance to chromatin. In contrast, Hron and colleagues demonstrated that SAP deficiency ameliorated all clinical features of disease including GN, when assessed at 5 months in the pristine-induced lupus model (110). As seen in our model, SAP deficiency mediated a delay in the kinetics of the autoantibody production that could potentially explain this discrepancy. Furthermore, it is unclear which haplotype the *Ly108* allele was derived from in this induced system.

SAP modulates a spectrum of immune functions, which includes playing a crucial role in NKT cell development, T_H2 cytokine production, and the establishment of long-term immunity (86, 99, 106-108, 111). Despite the fact that SAP knockout mice have a primary defect in the generation of long-lived plasma memory cells and total loss of NKT cells, these mice still generated high titer autoantibody in the presence of *Sle1b*. This strongly suggests that the role of these populations plays a minor role in the breach in tolerance, but might significantly impact ANA penetrance.

Although we have shown that the IL-4 deficiency is not necessary for the breach in tolerance mediated by *Sle1b*, the defect may, however, play a role in augmenting the loss in tolerance since B6.*Sle1bSap*KO mice have a reduced ANA penetrance compared to B6.*Sle1b*. Deficiency in SAP completely abolished IL-4 production when CD4 T cells were stimulated, suggesting that SAP is necessary for this immune response. Based on the CD4 T cell phenotypes seen in knockouts of *Ly9*, *Slam*, and *Ly108*, the absence of any of these molecules also leads to decreased IL-4 production. All of them have ITSMs in their cytoplasmic tails and the capacity to recruit SAP. However, using a 129 congenic strain carrying the *Sle1b* haplotype, we were able to recapitulate the reduction in IL-4 levels in the presence of all of these molecules. This suggests that the *Sle1b* haplotype harbors a dysfunctional allele for *Ly9*, *Slam*, and/or *Ly108*. We have previously established that in CD4 T cells, *Ly108* isoform expression is significantly different from B6, whereas *Slam* and *Ly9* did not show such a significant difference. This difference in isoform utilization could potentially change the number of ITSMs that are available for SAP binding, though Ly108 engagement of either isoform mediated similar effects. Sequence polymorphisms causing amino acid changes were present in *Ly9*, but not in *Slam* and *Ly108*. In particular, one of the mutations, a glycine to a glutamic acid, lies one amino acid away from the first TxYxxV/I motif in Ly9, and could potentially interfere with the proper recruitment of SAP to its cytoplasmic tail. Taken together, we speculate that the differences in the sequence of *Ly9*, and in *Ly108* isoform expression, result in dysfunctional alleles that could potentially mediate the IL-4 phenotype, though it does not preclude the contributions of other

differences in alleles of the SLAM family.

It is important to note that all the above-described SLAM family knockouts were all produced by deleting the gene in a 129 ES cell line and subsequent backcrossing to B6. Since the 129 genome shares the same *Sle1b* haplotype that has lupus-promoting alleles, it is difficult to distinguish the consequences of the deleted gene from those arising from the dysfunctional alleles on the B6 background. Furthermore, we speculate that CD84 might also be playing a role. Although a knockout has not yet been generated, CD84 is expressed in CD4 T cells and has the ability to bind SAP. It is possible that it is the combination of all four of these genes, *Ly9*, *Slam*, *Cd84*, and *Ly108*, that is necessary to recruit an adequate amount of SAP to the immune synapse to mediate signaling for IL-4 production. By generating deletions using a B6 ES cell line, we could eliminate the problem of the contributions from the haplotype, and accurately distinguish each gene's function on the B6 background.

ALLELIC MOUSE MODELS

Studies of complex genetic diseases commonly lead to mapping susceptibility loci to high-density haplotype blocks. This makes it very difficult to conclusively identify the causative gene and the specific polymorphism(s). Multiple groups have used BAC transgenic rescue strategies to successfully pinpoint the region harboring the causal gene (129-131). Similarly, we were also able to effectively narrow our ~940 kb region by complementing the genetic defect in *Sle1b* with a single BAC carrying the *Cd84* and *Ly108* alleles of B6. We then went

on to generate BAC transgenic mice carrying the different allelic variants of Ly108 on the reciprocal genetic backgrounds in order to look for disease suppression and potentiation.

These *in vivo* models demonstrate, for the first time, the ability to identify causative allelic variants. Previous studies have used BAC transgenic strategies to complement a phenotype resulting from the complete loss of function of the causative gene. Except in rare cases, complex genetic disorders are not a consequence of the complete loss of function of a gene, nor are they a result of huge changes in gene expression, as seen in some conventional transgenic mouse strains that develop lupus-like phenotypes. Instead, the genes mediating complex diseases, such as SLE, are commonly a result of subtle changes in function, such as small modifications in gene expression and isoform utilization (as in the case with Ly108), or minor protein structural changes. It is thus imperative to have the ability to fine-adjust the function of a gene to recapitulate these subtle variations. BACs serve as the ideal tool for such manipulations, due to their capacity to carry most, if not all, of the necessary regulatory elements to achieve accurate and precise expression of a given gene. Furthermore, with the availability of BAC libraries derived from various mouse strains, we can select both the causative and resistant alleles of the gene and generate transgenic models. In addition, we can use BAC modification strategies to conclusively identify the 'necessary and sufficient' polymorphism once a causal gene is localized. Such studies will not only serve to aid *in vivo* gene identification, but also as to generate systems with which to study the allelic differences in a gene's function on specific genetic backgrounds.

One caveat with the above described strategy is the presence of both the transgenic and endogenous alleles, which can create competition between the susceptible and non-susceptible alleles. As we have observed in our Ly108 model, the expression of the 129-derived BAC transgene did not fully reconstitute the isoform ratio present in *Sle1b* mice. This could explain why we were unable to fully recapitulate the penetrance of ANA production. The ideal transgenic system with which to elucidate the consequences of allelic variations would be to introduce the transgene into the genome of a mouse that completely lacks the gene, such that expression of the gene in the transgenic strain comes from only the 'susceptible' or 'resistant' allele. Our attempts to generate a Ly108 knockout mouse identified two positive clones that underwent homologous recombination. To date, we have not obtained germline transmission from these positive clones. However, we have subcloned the positive clones and are hopeful that one of them will transmit. Despite not having generated a knockout of Ly108, our allelic model does induce ANA production, even in the presence of the 'resistant' allele. Current work in our lab is focused on using a modified-BAC targeting approach to replace the endogenous allele of 129 with the B6 allele to look for suppression of autoantibody production. If suppression is mediated, we will be able to further determine whether the loss of expression of a specific isoform, Ly108-2, can induce ANA production.

Although Ly108 mediates the breach in tolerance, other genes within the family may also be contributing to the phenotype, such as *Cd84*. To fully understand how the different alleles function *in vivo*, it is critical to study the function of each gene individually. This entails first

the generation of a mouse lacking the entire SLAM family. To this end, we have generated two targeting constructs containing LoxP sites that will flank the SLAM family. One clone has been identified that has undergone homologous recombination of both the targeting vectors. We have injected this clone and are waiting for chimeric progeny. Once a deletion of the family has been generated, we can then express each individual SLAM family member, or combinations of them, from the various BAC transgenic lines we already have. We can also introduce the allelic variants of the family to understand how subtle changes affect their different roles in immune function. Although the system is quite cumbersome and time-consuming to establish, it is the only means possible to study *in vivo* functions of these genes, as well as their allelic variants in isolation and in various combinations. Furthermore, since lupus cannot be 'recapitulated in a dish', *in vivo* identification is the only means by which to conclusively identify the causal variant.

SUMMARY

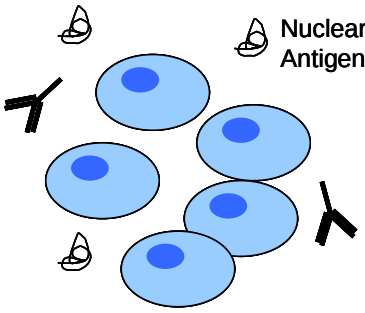
SLE is a polygenic disease and there is a wide body of evidence indicating that genetic predisposition is the major component dictating susceptibility. It is the prototypic systemic autoimmune disease with a highly heterogeneous clinical presentation. However, one common feature shared by most patients is the production of ANAs. It has been demonstrated that autoantibody production precedes the onset of clinical disease, potentially defining a preclinical period in which we can prevent disease progression before it becomes irreversible. Furthermore, ANA serves as a biomarker not only for SLE, but also other systemic autoimmune diseases, and represents a state of genetic predisposition and increased

probability of disease development. Understanding what initiates the development of autoantibodies may help us to elucidate common pathways mediating autoimmunity that could one day serve as potential therapeutic targets.

We have presented here the identification of *Ly108* as a causative gene in mediating the loss in tolerance. Furthermore, we have identified other contributing genes that work with *Ly108* to enhance autoantibody production. Figure 21 illustrates the changes phenotypically see in the mouse models, and Figure 22 depicts subtle cellular changes that are needed to recapitulate the *Sle1b* phenotype.

Development of ANA

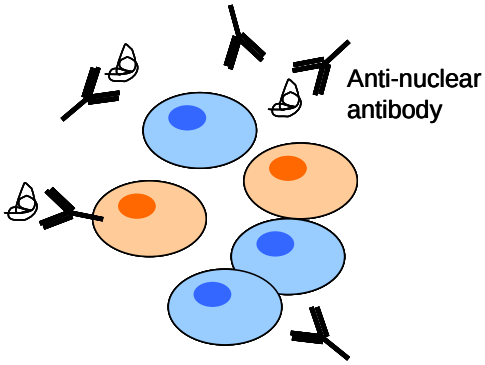
Normal Tolerized Cells



***Sle1b* Allele of Ly108**

and/or CD84?

Breach in Tolerance to Nuclear Antigens



SAP and/or *Sle1b* Alleles of 2B4, Ly9, Cs1,

and/or CD84

High Penetrance High Titer ANA

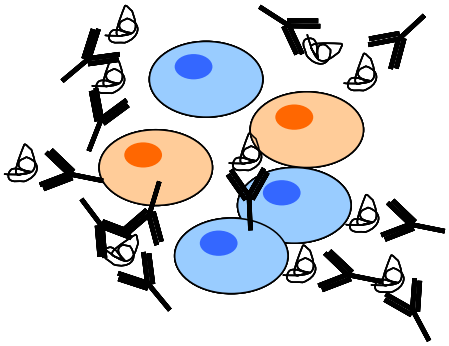


Figure 21. SLAM family alleles derived from B6.*Sle1b* mediated ANA Development.

The schematic diagrams the contribution of the SLAM family at two different stages to mediate fully penetrant, high titer ANA production present in B6.*Sle1b* mice. *Ly108* is sufficient and necessary for the breach in tolerance. Additional genes in SLAM family signaling, SAP, as well as other members potentiate the anti-chromatin production.

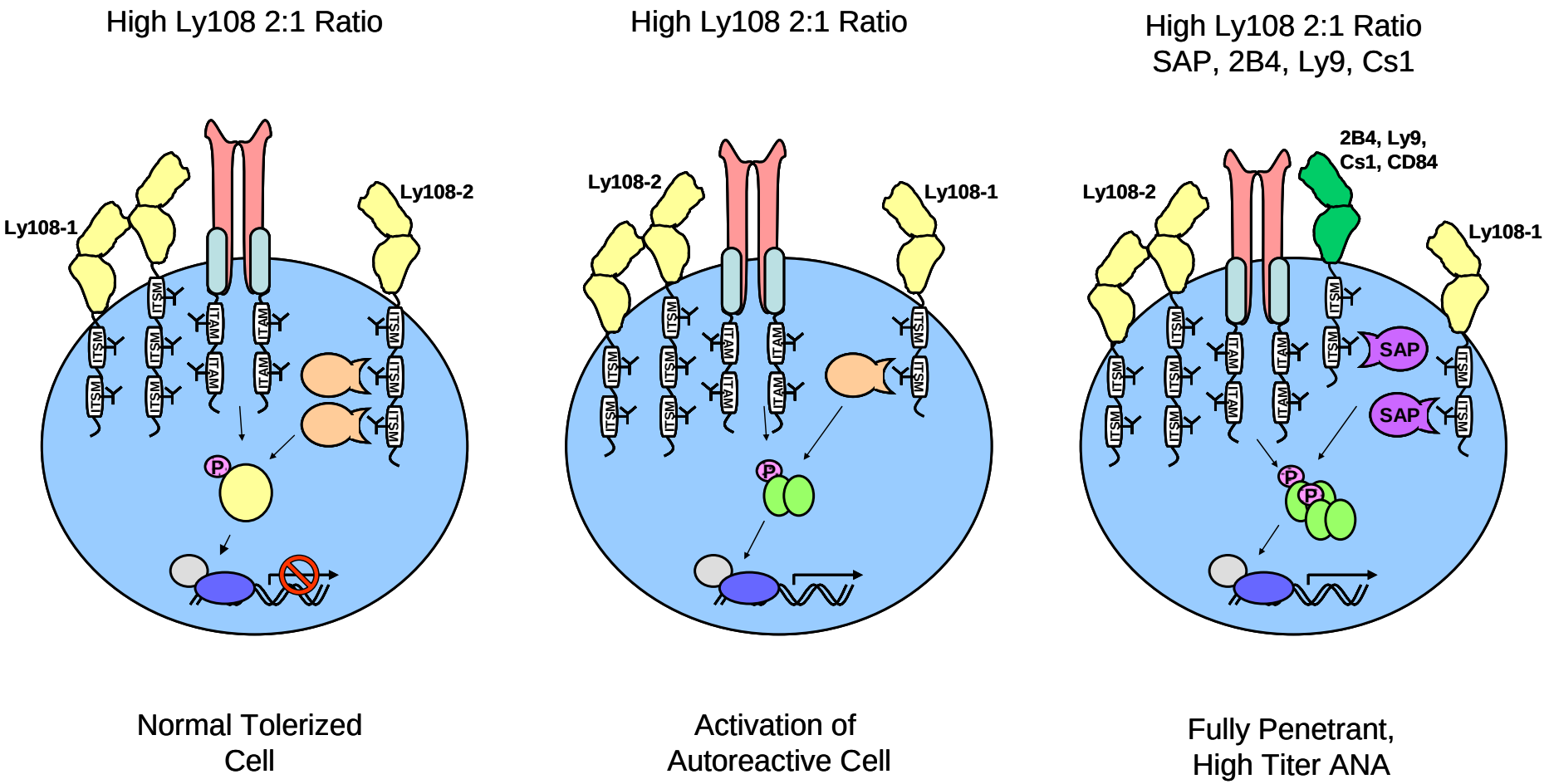


Figure 22. Subtle changes in the SLAM family mediate the *Sle1b* phenotype.

This figure depicts the players responsible for development of ANA. Changes in the isoform ratio of Ly108 mediate the loss in tolerance of an autoreactive cell. Other SLAM family members and SAP contribute to augmenting the cellular changes that lead to high titer ANA production.

BIBLIOGRAPHY

1. Sinha, A. A., M. T. Lopez, and H. O. McDevitt. 1990. Autoimmune diseases: the failure of self tolerance. *Science* 248:1380-1388.

2. Jacobson, D. L., S. J. Gange, N. R. Rose, and N. M. Graham. 1997. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. *Clin Immunol Immunopathol* 84:223-243.

3. Arbuckle, M. R., M. T. McClain, M. V. Rubertone, R. H. Scofield, G. J. Dennis, J. A. James, and J. B. Harley. 2003. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med* 349:1526-1533.

4. Kasper, D. L., E. Braunwald, A. Fauci, S. Hauser, D. Longo, and J. L. Jameson. 2004. *Disorders of the Immune System, Connective Tissue, and Joints*. McGraw-Hill Professional.

5. Clark, S. H. 2005. Animal models in scleroderma. *Curr Rheumatol Rep* 7:150-155.

6. Hoffman, R. W., M. A. Alspaugh, K. S. Waggie, J. B. Durham, and S. E. Walker. 1984. Sjogren's syndrome in MRL/l and MRL/n mice. *Arthritis Rheum* 27:157-165.

7. Hu, Y., Y. Nakagawa, K. R. Purushotham, and M. G. Humphreys-Beher. 1992. Functional changes in salivary glands of autoimmune disease-prone NOD mice. *Am J Physiol* 263:E607-614.

8. Hochberg, M. C., D. L. Perlmutter, T. A. Medsger, V. Steen, M. H. Weisman, B.

White, and F. M. Wigley. 1995. Prevalence of self-reported physician-diagnosed systemic lupus erythematosus in the USA. *Lupus* 4:454-456.

9. Deapen, D., A. Escalante, L. Weinrib, D. Horwitz, B. Bachman, P. Roy-Burman, A. Walker, and T. M. Mack. 1992. A revised estimate of twin concordance in systemic lupus erythematosus. *Arthritis Rheum* 35:311-318.

10. Block, S. R., J. B. Winfield, M. D. Lockshin, W. A. D'Angelo, M. E. Weksler, M. Fotino, and C. L. Christian. 1975. Proceedings: Twin studies in systemic lupus erythematosus (SLE). *Arthritis Rheum* 18:285.

11. Wakeland, E. K., K. Liu, R. R. Graham, and T. W. Behrens. 2001. Delineating the genetic basis of systemic lupus erythematosus. *Immunity* 15:397-408.

12. Soriano, E. R., and N. J. McHugh. 2004. *Overlap syndromes in adults and children*. Oxford University Press, New York.

13. Torfs, C. P., M. C. King, B. Huey, J. Malmgren, and F. C. Grumet. 1986. Genetic interrelationship between insulin-dependent diabetes mellitus, the autoimmune thyroid diseases, and rheumatoid arthritis. *Am J Hum Genet* 38:170-187.

14. Ginn, L. R., J. P. Lin, P. H. Plotz, S. J. Bale, R. L. Wilder, A. Mbauya, and F. W. Miller. 1998. Familial autoimmunity in pedigrees of idiopathic inflammatory myopathy patients suggests common genetic risk factors for many autoimmune diseases. *Arthritis Rheum* 41:400-405.

15. Lin, J. P., J. M. Cash, S. Z. Doyle, S. Peden, K. Kanik, C. I. Amos, S. J. Bale, and R. L. Wilder. 1998. Familial clustering of rheumatoid arthritis with other autoimmune diseases. *Hum Genet* 103:475-482.

16. Broadley, S. A., J. Deans, S. J. Sawcer, D. Clayton, and D. A. Compston. 2000. Autoimmune disease in first-degree relatives of patients with multiple sclerosis. A UK survey. *Brain* 123 (Pt 6):1102-1111.

17. Criswell, L. A., K. A. Pfeiffer, R. F. Lum, B. Gonzales, J. Novitzke, M. Kern, K. L. Moser, A. B. Begovich, V. E. Carlton, W. Li, A. T. Lee, W. Ortmann, T. W. Behrens, and P. K. Gregersen. 2005. Analysis of families in the multiple autoimmune disease genetics consortium (MADGC) collection: the PTPN22 620W allele associates with multiple autoimmune phenotypes. *Am J Hum Genet* 76:561-571.

18. Rudofsky, U. H., B. D. Evans, S. L. Balaban, V. D. Mottironi, and A. E. Gabrielsen. 1993. Differences in expression of lupus nephritis in New Zealand mixed H-2z homozygous inbred strains of mice derived from New Zealand black and New Zealand white mice. Origins and initial characterization. *Lab Invest* 68:419-426.

19. Morel, L., and E. K. Wakeland. 1998. Susceptibility to lupus nephritis in the NZB/W model system. *Curr Opin Immunol* 10:718-725.

20. Morel, L., U. H. Rudofsky, J. A. Longmate, J. Schiffenbauer, and E. K. Wakeland. 1994. Polygenic control of susceptibility to murine systemic lupus erythematosus. *Immunity* 1:219-229.

21. Wakeland, E., L. Morel, K. Achey, M. Yui, and J. Longmate. 1997. Speed congenics: a classic technique in the fast lane (relatively speaking). *Immunol Today* 18:472-477.

22. Morel, L., B. P. Croker, K. R. Blenman, C. Mohan, G. Huang, G. Gilkeson, and E. K. Wakeland. 2000. Genetic reconstitution of systemic lupus erythematosus immunopathology with polycongenic murine strains. *Proc Natl Acad Sci U S A* 97:6670-6675.

23. Mohan, C., E. Alas, L. Morel, P. Yang, and E. K. Wakeland. 1998. Genetic dissection of SLE pathogenesis. Sle1 on murine chromosome 1 leads to a selective loss of tolerance to H2A/H2B/DNA subnucleosomes. *J Clin Invest* 101:1362-1372.

24. Sobel, E. S., C. Mohan, L. Morel, J. Schiffenbauer, and E. K. Wakeland. 1999. Genetic dissection of SLE pathogenesis: adoptive transfer of Sle1 mediates the loss of tolerance by bone marrow-derived B cells. *J Immunol* 162:2415-2421.

25. Sobel, E. S., M. Satoh, Y. Chen, E. K. Wakeland, and L. Morel. 2002. The major murine systemic lupus erythematosus susceptibility locus Sle1 results in abnormal functions of both B and T cells. *J Immunol* 169:2694-2700.

26. Morel, L., K. R. Blenman, B. P. Croker, and E. K. Wakeland. 2001. The major murine systemic lupus erythematosus susceptibility locus, Sle1, is a cluster of functionally related genes. *Proc Natl Acad Sci U S A* 98:1787-1792.

27. Wandstrat, A. E., C. Nguyen, N. Limaye, A. Y. Chan, S. Subramanian, X. H. Tian, Y. S. Yim, A. Pertsemlidis, H. R. Garner, Jr., L. Morel, and E. K. Wakeland. 2004. Association of extensive polymorphisms in the SLAM/CD2 gene cluster with murine lupus. *Immunity* 21:769-780.

28. Nguyen, C. 2005. Haplotype-specific Effects of the SLAM/CD2 Family on the

Immune Response. In *Immunology*. University of Texas Southwestern Medical Center, Dallas. 170.

29. Limaye, N. 2005. Common Alleles of the SLAM/CD2 Family are Associated with Murine Lupus. In *Center for Immunology*. University of Texas Southwestern Medical Center, Dallas. 225.

30. Garni-Wagner, B. A., A. Purohit, P. A. Mathew, M. Bennett, and V. Kumar. 1993. A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells. *J Immunol* 151:60-70.

31. Mathew, P. A., B. A. Garni-Wagner, K. Land, A. Takashima, E. Stoneman, M. Bennett, and V. Kumar. 1993. Cloning and characterization of the 2B4 gene encoding a molecule associated with non-MHC-restricted killing mediated by activated natural killer cells and T cells. *J Immunol* 151:5328-5337.

32. Nakajima, H., M. Cella, H. Langen, A. Friedlein, and M. Colonna. 1999. Activating interactions in human NK cell recognition: the role of 2B4-CD48. *Eur J Immunol* 29:1676-1683.

33. Kiel, M. J., O. H. Yilmaz, T. Iwashita, O. H. Yilmaz, C. Terhorst, and S. J. Morrison. 2005. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 121:1109-1121.

34. Stepp, S. E., J. D. Schatzle, M. Bennett, V. Kumar, and P. A. Mathew. 1999. Gene structure of the murine NK cell receptor 2B4: presence of two alternatively spliced isoforms with distinct cytoplasmic domains. *Eur J Immunol* 29:2392-

2399.

35. Tangye, S. G., S. Lazetic, E. Woollatt, G. R. Sutherland, L. L. Lanier, and J. H. Phillips. 1999. Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP. *J Immunol* 162:6981-6985.

36. Chen, R., F. Relouzat, R. Roncagalli, A. Aoukaty, R. Tan, S. Latour, and A. Veillette. 2004. Molecular dissection of 2B4 signaling: implications for signal transduction by SLAM-related receptors. *Mol Cell Biol* 24:5144-5156.

37. Eissmann, P., L. Beauchamp, J. Wooters, J. C. Tilton, E. O. Long, and C. Watzl. 2005. Molecular basis for positive and negative signaling by the natural killer cell receptor 2B4 (CD244). *Blood* 105:4722-4729.

38. Schatzle, J. D., S. Sheu, S. E. Stepp, P. A. Mathew, M. Bennett, and V. Kumar. 1999. Characterization of inhibitory and stimulatory forms of the murine natural killer cell receptor 2B4. *Proc Natl Acad Sci U S A* 96:3870-3875.

39. Bloch-Queyrat, C., M. C. Fondaneche, R. Chen, L. Yin, F. Relouzat, A. Veillette, A. Fischer, and S. Latour. 2005. Regulation of natural cytotoxicity by the adaptor SAP and the Src-related kinase Fyn. *J Exp Med* 202:181-192.

40. Mooney, J. M., J. Klem, C. Wulfing, L. A. Mijares, P. L. Schwartzberg, M. Bennett, and J. D. Schatzle. 2004. The murine NK receptor 2B4 (CD244) exhibits inhibitory function independent of signaling lymphocytic activation molecule-associated protein expression. *J Immunol* 173:3953-3961.

41. Lee, K. M., M. E. McNerney, S. E. Stepp, P. A. Mathew, J. D. Schatzle, M.

Bennett, and V. Kumar. 2004. 2B4 acts as a non-major histocompatibility complex binding inhibitory receptor on mouse natural killer cells. *J Exp Med* 199:1245-1254.

42. McNerney, M. E., D. Guzior, and V. Kumar. 2005. 2B4 (CD244)-CD48 interactions provide a novel MHC class I-independent system for NK-cell self-tolerance in mice. *Blood* 106:1337-1340.

43. Gao, N., P. Schwartzberg, J. A. Wilder, B. R. Blazar, and D. Yuan. 2006. B cell induction of IL-13 expression in NK cells: Role of CD244 and SLAM-associated protein. *J Immunol* 176:2758-2764.

44. Gao, N., T. Dang, W. A. Dunnick, J. T. Collins, B. R. Blazar, and D. Yuan. 2005. Receptors and counterreceptors involved in NK-B cell interactions. *J Immunol* 174:4113-4119.

45. Mathieson, B. J., S. O. Sharrow, K. Bottomly, and B. J. Fowlkes. 1980. Ly 9, an alloantigenic marker of lymphocyte differentiation. *J Immunol* 125:2127-2136.

46. Sandrin, M. S., T. P. Gumley, M. M. Henning, H. A. Vaughan, L. J. Gonez, J. A. Trapani, and I. F. McKenzie. 1992. Isolation and characterization of cDNA clones for mouse Ly-9. *J Immunol* 149:1636-1641.

47. Sandrin, M. S., M. M. Henning, M. F. Lo, E. Baker, G. R. Sutherland, and I. F. McKenzie. 1996. Isolation and characterization of cDNA clones for Humly9: the human homologue of mouse Ly9. *Immunogenetics* 43:13-19.

48. Sayos, J., M. Martin, A. Chen, M. Simarro, D. Howie, M. Morra, P. Engel, and C. Terhorst. 2001. Cell surface receptors Ly-9 and CD84 recruit the X-linked

lymphoproliferative disease gene product SAP. *Blood* 97:3867-3874.

49. Martin, M., X. Romero, M. A. de la Fuente, V. Tovar, N. Zapater, E. Esplugues, P. Pizcueta, J. Bosch, and P. Engel. 2001. CD84 functions as a homophilic adhesion molecule and enhances IFN-gamma secretion: adhesion is mediated by Ig-like domain 1. *J Immunol* 167:3668-3676.

50. Romero, X., N. Zapater, M. Calvo, S. G. Kalko, M. A. de la Fuente, V. Tovar, C. Ockeloen, P. Pizcueta, and P. Engel. 2005. CD229 (Ly9) lymphocyte cell surface receptor interacts homophilically through its N-terminal domain and relocalizes to the immunological synapse. *J Immunol* 174:7033-7042.

51. Tovar, V., M. A. de la Fuente, P. Pizcueta, J. Bosch, and P. Engel. 2000. Gene structure of the mouse leukocyte cell surface molecule Ly9. *Immunogenetics* 51:788-793.

52. Graham, D. B., M. P. Bell, M. M. McCausland, C. J. Huntoon, J. van Deursen, W. A. Faubion, S. Crotty, and D. J. McKean. 2006. Ly9 (CD229)-deficient mice exhibit T cell defects yet do not share several phenotypic characteristics associated with SLAM- and SAP-deficient mice. *J Immunol* 176:291-300.

53. Simarro, M., A. Lanyi, D. Howie, F. Poy, J. Bruggeman, M. Choi, J. Sumegi, M. J. Eck, and C. Terhorst. 2004. SAP increases FynT kinase activity and is required for phosphorylation of SLAM and Ly9. *Int Immunol* 16:727-736.

54. Vinuesa, C. G., M. C. Cook, C. Angelucci, V. Athanasopoulos, L. Rui, K. M. Hill, D. Yu, H. Domaschenz, B. Whittle, T. Lambe, I. S. Roberts, R. R. Copley, J. I. Bell, R. J. Cornall, and C. C. Goodnow. 2005. A RING-type ubiquitin ligase

family member required to repress follicular helper T cells and autoimmunity. *Nature* 435:452-458.

55. Chtanova, T., S. G. Tangye, R. Newton, N. Frank, M. R. Hodge, M. S. Rolph, and C. R. Mackay. 2004. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J Immunol* 173:68-78.

56. Boles, K. S., and P. A. Mathew. 2001. Molecular cloning of CS1, a novel human natural killer cell receptor belonging to the CD2 subset of the immunoglobulin superfamily. *Immunogenetics* 52:302-307.

57. Bouchon, A., M. Cella, H. L. Grierson, J. I. Cohen, and M. Colonna. 2001. Activation of NK cell-mediated cytotoxicity by a SAP-independent receptor of the CD2 family. *J Immunol* 167:5517-5521.

58. Kumaresan, P. R., W. C. Lai, S. S. Chuang, M. Bennett, and P. A. Mathew. 2002. CS1, a novel member of the CD2 family, is homophilic and regulates NK cell function. *Mol Immunol* 39:1-8.

59. Tovar, V., J. del Valle, N. Zapater, M. Martin, X. Romero, P. Pizcueta, J. Bosch, C. Terhorst, and P. Engel. 2002. Mouse novel Ly9: a new member of the expanding CD150 (SLAM) family of leukocyte cell-surface receptors. *Immunogenetics* 54:394-402.

60. Roncagalli, R., J. E. Taylor, S. Zhang, X. Shi, R. Chen, M. E. Cruz-Munoz, L. Yin, S. Latour, and A. Veillette. 2005. Negative regulation of natural killer cell function by EAT-2, a SAP-related adaptor. *Nat Immunol* 6:1002-1010.

61. Tassi, I., and M. Colonna. 2005. The cytotoxicity receptor CRACC (CS-1) recruits EAT-2 and activates the PI3K and phospholipase Cgamma signaling pathways in human NK cells. *J Immunol* 175:7996-8002.

62. Gonzalez-Cabrero, J., C. J. Wise, Y. Latchman, G. J. Freeman, A. H. Sharpe, and H. Reiser. 1999. CD48-deficient mice have a pronounced defect in CD4(+) T cell activation. *Proc Natl Acad Sci U S A* 96:1019-1023.

63. Reiser, H. 1990. sgp-60, a signal-transducing glycoprotein concerned with T cell activation through the T cell receptor/CD3 complex. *J Immunol* 145:2077-2086.

64. Kato, K., M. Koyanagi, H. Okada, T. Takanashi, Y. W. Wong, A. F. Williams, K. Okumura, and H. Yagita. 1992. CD48 is a counter-receptor for mouse CD2 and is involved in T cell activation. *J Exp Med* 176:1241-1249.

65. Thorley-Lawson, D. A., R. T. Schooley, A. K. Bhan, and L. M. Nadler. 1982. Epstein-Barr virus superinduces a new human B cell differentiation antigen (B-LAST 1) expressed on transformed lymphoblasts. *Cell* 30:415-425.

66. Arvieux, J., A. C. Willis, and A. F. Williams. 1986. MRC OX-45 antigen: a leucocyte/endothelium rat membrane glycoprotein of 45,000 molecular weight. *Mol Immunol* 23:983-990.

67. Wong, Y. W., A. F. Williams, S. F. Kingsmore, and M. F. Seldin. 1990. Structure, expression, and genetic linkage of the mouse BCM1 (OX45 or Blast-1) antigen. Evidence for genetic duplication giving rise to the BCM1 region on mouse chromosome 1 and the CD2/LFA3 region on mouse chromosome 3. *J Exp Med* 171:2115-2130.

68. Brown, M. H., K. Boles, P. A. van der Merwe, V. Kumar, P. A. Mathew, and A. N. Barclay. 1998. 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J Exp Med* 188:2083-2090.

69. Malaviya, R., Z. Gao, K. Thankavel, P. A. van der Merwe, and S. N. Abraham. 1999. The mast cell tumor necrosis factor alpha response to FimH-expressing Escherichia coli is mediated by the glycosylphosphatidylinositol-anchored molecule CD48. *Proc Natl Acad Sci U S A* 96:8110-8115.

70. Howie, D., M. Simarro, J. Sayos, M. Guirado, J. Sancho, and C. Terhorst. 2002. Molecular dissection of the signaling and costimulatory functions of CD150 (SLAM): CD150/SAP binding and CD150-mediated costimulation. *Blood* 99:957-965.

71. Theil, D., C. Farina, and E. Meinl. 2005. Differential expression of CD150 (SLAM) on monocytes and macrophages in chronic inflammatory contexts: abundant in Crohn's disease, but not in multiple sclerosis. *J Clin Pathol* 58:110-111.

72. Nanda, N., P. Andre, M. Bao, K. Clauser, F. Deguzman, D. Howie, P. B. Conley, C. Terhorst, and D. R. Phillips. 2005. Platelet aggregation induces platelet aggregate stability via SLAM family receptor signaling. *Blood* 106:3028-3034.

73. Castro, A. G., T. M. Hauser, B. G. Cocks, J. Abrams, S. Zurawski, T. Churakova, F. Zonin, D. Robinson, S. G. Tangye, G. Aversa, K. E. Nichols, J. E. de Vries, L. L. Lanier, and A. O'Garra. 1999. Molecular and functional characterization of mouse signaling lymphocytic activation molecule (SLAM): differential expression and responsiveness in Th1 and Th2 cells. *J Immunol* 163:5860-5870.

74. Tatsuo, H., N. Ono, K. Tanaka, and Y. Yanagi. 2000. SLAM (CDw150) is a cellular receptor for measles virus. *Nature* 406:893-897.

75. Wang, N., M. Morra, C. Wu, C. Gullo, D. Howie, T. Coyle, P. Engel, and C. Terhorst. 2001. CD150 is a member of a family of genes that encode glycoproteins on the surface of hematopoietic cells. *Immunogenetics* 53:382-394.

76. Fisher, R. C., J. D. Lovelock, and E. W. Scott. 1999. A critical role for PU.1 in homing and long-term engraftment by hematopoietic stem cells in the bone marrow. *Blood* 94:1283-1290.

77. Mavaddat, N., D. W. Mason, P. D. Atkinson, E. J. Evans, R. J. Gilbert, D. I. Stuart, J. A. Fennelly, A. N. Barclay, S. J. Davis, and M. H. Brown. 2000. Signaling lymphocytic activation molecule (CDw150) is homophilic but self-associates with very low affinity. *J Biol Chem* 275:28100-28109.

78. Sayos, J., C. Wu, M. Morra, N. Wang, X. Zhang, D. Allen, S. van Schaik, L. Notarangelo, R. Geha, M. G. Roncarolo, H. Oettgen, J. E. De Vries, G. Aversa, and C. Terhorst. 1998. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature* 395:462-469.

79. Shlapatska, L. M., S. V. Mikhalap, A. G. Berdova, O. M. Zelensky, T. J. Yun, K. E. Nichols, E. A. Clark, and S. P. Sidorenko. 2001. CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A. *J Immunol* 166:5480-5487.

80. Mikhalap, S. V., L. M. Shlapatska, O. V. Yurchenko, M. Y. Yurchenko, G. G. Berdova, K. E. Nichols, E. A. Clark, and S. P. Sidorenko. 2004. The adaptor protein SH2D1A regulates signaling through CD150 (SLAM) in B cells. *Blood* 104:4063-4070.

81. Howie, D., S. Okamoto, S. Rietdijk, K. Clarke, N. Wang, C. Gullo, J. P. Bruggeman, S. Manning, A. J. Coyle, E. Greenfield, V. Kuchroo, and C. Terhorst. 2002. The role of SAP in murine CD150 (SLAM)-mediated T-cell proliferation and interferon gamma production. *Blood* 100:2899-2907.

82. Cocks, B. G., C. C. Chang, J. M. Carballido, H. Yssel, J. E. de Vries, and G. Aversa. 1995. A novel receptor involved in T-cell activation. *Nature* 376:260-263.

83. Wang, N., A. Satoskar, W. Faubion, D. Howie, S. Okamoto, S. Feske, C. Gullo, K. Clarke, M. R. Sosa, A. H. Sharpe, and C. Terhorst. 2004. The cell surface receptor SLAM controls T cell and macrophage functions. *J Exp Med* 199:1255-1264.

84. Czar, M. J., E. N. Kersh, L. A. Mijares, G. Lanier, J. Lewis, G. Yap, A. Chen, A. Sher, C. S. Duckett, R. Ahmed, and P. L. Schwartzberg. 2001. Altered lymphocyte responses and cytokine production in mice deficient in the X-linked lymphoproliferative disease gene SH2D1A/DSHP/SAP. *Proc Natl Acad Sci U S A* 98:7449-7454.

85. Wu, C., K. B. Nguyen, G. C. Pien, N. Wang, C. Gullo, D. Howie, M. R. Sosa, M. J. Edwards, P. Borrow, A. R. Satoskar, A. H. Sharpe, C. A. Biron, and C. Terhorst. 2001. SAP controls T cell responses to virus and terminal differentiation

of TH2 cells. *Nat Immunol* 2:410-414.

86. Cannons, J. L., L. J. Yu, B. Hill, L. A. Mijares, D. Dombroski, K. E. Nichols, A. Antonellis, G. A. Koretzky, K. Gardner, and P. L. Schwartzberg. 2004. SAP regulates T(H)2 differentiation and PKC-theta-mediated activation of NF-kappaB1. *Immunity* 21:693-706.

87. Punnonen, J., B. G. Cocks, J. M. Carballido, B. Bennett, D. Peterson, G. Aversa, and J. E. de Vries. 1997. Soluble and membrane-bound forms of signaling lymphocytic activation molecule (SLAM) induce proliferation and Ig synthesis by activated human B lymphocytes. *J Exp Med* 185:993-1004.

88. Tangye, S. G., B. C. van de Weerdt, D. T. Avery, and P. D. Hodgkin. 2002. CD84 is up-regulated on a major population of human memory B cells and recruits the SH2 domain containing proteins SAP and EAT-2. *Eur J Immunol* 32:1640-1649.

89. de la Fuente, M. A., V. Tovar, P. Pizcueta, M. Nadal, J. Bosch, and P. Engel. 1999. Molecular cloning, characterization, and chromosomal localization of the mouse homologue of CD84, a member of the CD2 family of cell surface molecules. *Immunogenetics* 49:249-255.

90. Tangye, S. G., K. E. Nichols, N. J. Hare, and B. C. van de Weerdt. 2003. Functional requirements for interactions between CD84 and Src homology 2 domain-containing proteins and their contribution to human T cell activation. *J Immunol* 171:2485-2495.

91. Bottino, C., M. Falco, S. Parolini, E. Marcenaro, R. Augugliaro, S. Sivori, E. Landi, R. Biassoni, L. D. Notarangelo, L. Moretta, and A. Moretta. 2001. NTB-A

[correction of GNTB-A], a novel SH2D1A-associated surface molecule contributing to the inability of natural killer cells to kill Epstein-Barr virus-infected B cells in X-linked lymphoproliferative disease. *J Exp Med* 194:235-246.

92. Peck, S. R., and H. E. Ruley. 2000. Ly108: a new member of the mouse CD2 family of cell surface proteins. *Immunogenetics* 52:63-72.

93. Fraser, C. C., D. Howie, M. Morra, Y. Qiu, C. Murphy, Q. Shen, J. C. Gutierrez-Ramos, A. Coyle, G. A. Kingsbury, and C. Terhorst. 2002. Identification and characterization of SF2000 and SF2001, two new members of the immune receptor SLAM/CD2 family. *Immunogenetics* 53:843-850.

94. Falco, M., E. Marcenaro, E. Romeo, F. Bellora, D. Marras, F. Vely, G. Ferracci, L. Moretta, A. Moretta, and C. Bottino. 2004. Homophilic interaction of NTBA, a member of the CD2 molecular family: induction of cytotoxicity and cytokine release in human NK cells. *Eur J Immunol* 34:1663-1672.

95. Valdez, P. A., H. Wang, D. Seshasayee, M. van Lookeren Campagne, A. Gurney, W. P. Lee, and I. S. Grewal. 2004. NTB-A, a new activating receptor in T cells that regulates autoimmune disease. *J Biol Chem* 279:18662-18669.

96. Flaig, R. M., S. Stark, and C. Watzl. 2004. Cutting edge: NTB-A activates NK cells via homophilic interaction. *J Immunol* 172:6524-6527.

97. Howie, D., F. S. Laroux, M. Morra, A. R. Satoskar, L. E. Rosas, W. A. Faubion, A. Julien, S. Rietdijk, A. J. Coyle, C. Fraser, and C. Terhorst. 2005. Cutting edge: the SLAM family receptor Ly108 controls T cell and neutrophil functions. *J Immunol* 174:5931-5935.

98. Coffey, A. J., R. A. Brooksbank, O. Brandau, T. Oohashi, G. R. Howell, J. M. Bye, A. P. Cahn, J. Durham, P. Heath, P. Wray, R. Pavitt, J. Wilkinson, M. Leversha, E. Huckle, C. J. Shaw-Smith, A. Dunham, S. Rhodes, V. Schuster, G. Porta, L. Yin, P. Serafini, B. Sylla, M. Zollo, B. Franco, A. Bolino, M. Seri, A. Lanyi, J. R. Davis, D. Webster, A. Harris, G. Lenoir, G. de St Basile, A. Jones, B. H. Behloradsky, H. Achatz, J. Murken, R. Fassler, J. Sumegi, G. Romeo, M. Vaudin, M. T. Ross, A. Meindl, and D. R. Bentley. 1998. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nat Genet* 20:129-135.

99. Nichols, K. E., J. Hom, S. Y. Gong, A. Ganguly, C. S. Ma, J. L. Cannons, S. G. Tangye, P. L. Schwartzberg, G. A. Koretzky, and P. L. Stein. 2005. Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat Med* 11:340-345.

100. Al-Alem, U., C. Li, N. Forey, F. Relouzat, M. C. Fondaneche, S. V. Tavtigian, Z. Q. Wang, S. Latour, and L. Yin. 2005. Impaired Ig class switch in mice deficient for the X-linked lymphoproliferative disease gene Sap. *Blood* 106:2069-2075.

101. Morra, M., R. A. Barrington, A. C. Abadia-Molina, S. Okamoto, A. Julien, C. Gullo, A. Kalsy, M. J. Edwards, G. Chen, R. Spolski, W. J. Leonard, B. T. Huber, P. Borrow, C. A. Biron, A. R. Satoskar, M. C. Carroll, and C. Terhorst. 2005. Defective B cell responses in the absence of SH2D1A. *Proc Natl Acad Sci U S A* 102:4819-4823.

102. Latour, S., G. Gish, C. D. Helgason, R. K. Humphries, T. Pawson, and A. Veillette. 2001. Regulation of SLAM-mediated signal transduction by SAP, the

X-linked lymphoproliferative gene product. *Nat Immunol* 2:681-690.

103. Latour, S., R. Roncagalli, R. Chen, M. Bakinowski, X. Shi, P. L. Schwartzberg, D. Davidson, and A. Veillette. 2003. Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signalling in immune regulation. *Nat Cell Biol* 5:149-154.

104. Chan, B., A. Lanyi, H. K. Song, J. Griesbach, M. Simarro-Grande, F. Poy, D. Howie, J. Sumegi, C. Terhorst, and M. J. Eck. 2003. SAP couples Fyn to SLAM immune receptors. *Nat Cell Biol* 5:155-160.

105. Yin, L., U. Al-Alem, J. Liang, W. M. Tong, C. Li, M. Badiali, J. J. Medard, J. Sumegi, Z. Q. Wang, and G. Romeo. 2003. Mice deficient in the X-linked lymphoproliferative disease gene sap exhibit increased susceptibility to murine gammaherpesvirus-68 and hypo-gammaglobulinemia. *J Med Virol* 71:446-455.

106. Chung, B., A. Aoukaty, J. Dutz, C. Terhorst, and R. Tan. 2005. Signaling lymphocytic activation molecule-associated protein controls NKT cell functions. *J Immunol* 174:3153-3157.

107. Pasquier, B., L. Yin, M. C. Fondaneche, F. Relouzat, C. Bloch-Queyrat, N. Lambert, A. Fischer, G. de Saint-Basile, and S. Latour. 2005. Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. *J Exp Med* 201:695-701.

108. Davidson, D., X. Shi, S. Zhang, H. Wang, M. Nemer, N. Ono, S. Ohno, Y. Yanagi, and A. Veillette. 2004. Genetic evidence linking SAP, the X-linked lymphoproliferative gene product, to Src-related kinase FynT in T(H)2 cytokine

regulation. *Immunity* 21:707-717.

109. Chen, G., A. K. Tai, M. Lin, F. Chang, C. Terhorst, and B. T. Huber. 2005. Signaling lymphocyte activation molecule-associated protein is a negative regulator of the CD8 T cell response in mice. *J Immunol* 175:2212-2218.

110. Hron, J. D., L. Caplan, A. J. Gerth, P. L. Schwartzberg, and S. L. Peng. 2004. SH2D1A regulates T-dependent humoral autoimmunity. *J Exp Med* 200:261-266.

111. Crotty, S., E. N. Kersh, J. Cannons, P. L. Schwartzberg, and R. Ahmed. 2003. SAP is required for generating long-term humoral immunity. *Nature* 421:282-287.

112. Komori, H., H. Furukawa, S. Mori, M. R. Ito, M. Terada, M. C. Zhang, N. Ishii, N. Sakuma, M. Nose, and M. Ono. 2006. A signal adaptor SLAM-associated protein regulates spontaneous autoimmunity and Fas-dependent lymphoproliferation in MRL-Faslpr lupus mice. *J Immunol* 176:395-400.

113. Thompson, A. D., B. S. Braun, A. Arvand, S. D. Stewart, W. A. May, E. Chen, J. Korenberg, and C. Denny. 1996. EAT-2 is a novel SH2 domain containing protein that is up regulated by Ewing's sarcoma EWS/FLI1 fusion gene. *Oncogene* 13:2649-2658.

114. Calpe, S., E. Erdos, G. Liao, N. Wang, S. Rietdijk, M. Simarro, B. Scholtz, J. Mooney, C. H. Lee, M. S. Shin, E. Rajnavolgyi, J. Schatzle, H. C. Morse, 3rd, C. Terhorst, and A. Lanyi. 2006. Identification and characterization of two related murine genes, Eat2a and Eat2b, encoding single SH2-domain adapters. *Immunogenetics* 58:15-25.

115. Morra, M., J. Lu, F. Poy, M. Martin, J. Sayos, S. Calpe, C. Gullo, D. Howie, S. Rietdijk, A. Thompson, A. J. Coyle, C. Denny, M. B. Yaffe, P. Engel, M. J. Eck, and C. Terhorst. 2001. Structural basis for the interaction of the free SH2 domain EAT-2 with SLAM receptors in hematopoietic cells. *Embo J* 20:5840-5852.

116. Aho, K., M. Heliovaara, J. Maatela, T. Tuomi, and T. Palosuo. 1991. Rheumatoid factors antedating clinical rheumatoid arthritis. *J Rheumatol* 18:1282-1284.

117. Rantapaa-Dahlqvist, S., B. A. de Jong, E. Berglin, G. Hallmans, G. Wadell, H. Stenlund, U. Sundin, and W. J. van Venrooij. 2003. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum* 48:2741-2749.

118. Nielen, M. M., D. van Schaardenburg, H. W. Reesink, R. J. van de Stadt, I. E. van der Horst-Bruinsma, M. H. de Koning, M. R. Habibuw, J. P. Vandenbroucke, and B. A. Dijkmans. 2004. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. *Arthritis Rheum* 50:380-386.

119. Batstra, M. R., H. J. Aanstoot, and P. Herbrink. 2001. Prediction and diagnosis of type 1 diabetes using beta-cell autoantibodies. *Clin Lab* 47:497-507.

120. Liang, Z., C. Xie, C. Chen, D. Kreska, K. Hsu, L. Li, X. J. Zhou, and C. Mohan. 2004. Pathogenic profiles and molecular signatures of antinuclear autoantibodies rescued from NZM2410 lupus mice. *J Exp Med* 199:381-398.

121. Kubota, K., H. Katoh, K. Muguruma, and K. Koyama. 1999. Characterization of a surface membrane molecule expressed by natural killer cells in most inbred

mouse strains: monoclonal antibody C9.1 identifies an allelic form of the 2B4 antigen. *Immunology* 96:491-497.

122. Kumaresan, P. R., V. T. Huynh, and P. A. Mathew. 2000. Polymorphism in the 2B4 gene of inbred mouse strains. *Immunogenetics* 51:758-761.

123. Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins, and J. D. Thompson. 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* 31:3497-3500.

124. Clamp, M., J. Cuff, S. M. Searle, and G. J. Barton. 2004. The Jalview Java alignment editor. *Bioinformatics* 20:426-427.

125. Bygrave, A. E., K. L. Rose, J. Cortes-Hernandez, J. Warren, R. J. Rigby, H. T. Cook, M. J. Walport, T. J. Vyse, and M. Botto. 2004. Spontaneous autoimmunity in 129 and C57BL/6 mice-implications for autoimmunity described in gene-targeted mice. *PLoS Biol* 2:E243.

126. Nichols, K. E., D. P. Harkin, S. Levitz, M. Krainer, K. A. Kolquist, C. Genovese, A. Bernard, M. Ferguson, L. Zuo, E. Snyder, A. J. Buckler, C. Wise, J. Ashley, M. Lovett, M. B. Valentine, A. T. Look, W. Gerald, D. E. Housman, and D. A. Haber. 1998. Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome. *Proc Natl Acad Sci U S A* 95:13765-13770.

127. Mooney, J. 2005. Defining how polymorphisms at the SLAM family locus affect NK and T cell function. In *Immunology*. University of Texas Southwestern Medical Center, Dallas. 228.

128. Ma, C. S., S. Pittaluga, D. T. Avery, N. J. Hare, I. Maric, A. D. Klion, K. E.

Nichols, and S. G. Tangye. 2006. Selective generation of functional somatically mutated IgM+CD27+, but not Ig isotype-switched, memory B cells in X-linked lymphoproliferative disease. *J Clin Invest* 116:322-333.

129. Antoch, M. P., E. J. Song, A. M. Chang, M. H. Vitaterna, Y. Zhao, L. D. Wilsbacher, A. M. Sangoram, D. P. King, L. H. Pinto, and J. S. Takahashi. 1997. Functional identification of the mouse circadian Clock gene by transgenic BAC rescue. *Cell* 89:655-667.

130. Lee, S. H., A. Zafer, Y. de Repentigny, R. Kothary, M. L. Tremblay, P. Gros, P. Duplay, J. R. Webb, and S. M. Vidal. 2003. Transgenic expression of the activating natural killer receptor Ly49H confers resistance to cytomegalovirus in genetically susceptible mice. *J Exp Med* 197:515-526.

131. Wines, M. E., Y. Shi, M. Lindor, and B. C. Holdener. 2000. Physical localization of the mesoderm development (mesd) functional region. *Genomics* 68:322-329.

VITAE

Alice Yanan Chan was born in Lafayette, Indiana on January 19, 1979, the daughter of Yaung-Hwa Chan and Wen Sheng Chan. She has one younger brother Winston Chan. After completing her work at the Texas Academy of Math and Science, Denton, Texas in 1997, she entered the University of Texas at Austin, Texas. She received the degree of Bachelor of Science with a major in biology in May, 1999. In June, 1999 she entered the Medical Scientist Training Program at the University of Texas Southwestern Medical Center at Dallas.

Permanent Address: 3915 Windview Drive
Colleyville, Texas 76034