Functional dissection of lupus susceptibility loci on the New Zealand black mouse chromosome 1

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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“Functional dissection of lupus susceptibility loci on the New Zealand black mouse chromosome 1”

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ABSTRACT

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease with a strong and complex genetic basis. To dissect the function of the lupus susceptibility loci on New Zealand black (NZB) mouse chromosome 1, the lab had previously generated congenic mice with an introgressed homozygous NZB chromosome 1 intervals extending from ~35 or ~82 to 106 cM on the C57BL/6 background. Although both mouse strains made IgG anti-nuclear antibodies (ANAs), ANA titres and cellular activation were significantly higher in mice with the longer interval. These studies suggest the presence of two susceptibility genes. In this thesis I have sought to further characterize the cellular abnormalities and underlying genetic polymorphisms that produce them in these mice. Using mixed hematopoietic chimeric mice, with a mixture of tagged-B6 and congenic bone marrow I demonstrate that there are intrinsic B and T cell functional defects in chromosome 1 congenic mice. I further show that an intrinsic B cell defect is required for efficient recruitment of B cells into the spontaneous germinal centres and differentiation of autoantibody producing cells in these mice. To more precisely localize the susceptibility loci, I produced and characterized a number of additional subcongenic mouse strains. This revealed surprising genetic complexity with the presence of at least four lupus susceptibility loci and a suppressor locus on chromosome 1, several of which
appeared to impact on T cell function. Finally, I generated bicongenic mice carrying both NZB chromosome 1 and 13 intervals, hypothesizing that since these were two of the major intervals associated with autoimmune disease in NZB mice they would fully recapitulate the autoimmune phenotypes. Although this hypothesis was incorrect, several novel phenotypes developed including marked expansion of the plasmacytoid and myeloid dendritic cell compartments and increased BAFF and IgA autoantibody production. Although this expansion was associated with TLR hyper-responsiveness, disease severity remained mild, possibly due to the lack of IFN-α production, which appeared to be inhibited in these mice. Thus, lupus arises from immune defects affecting several cellular populations, which are the product of multiple genetic polymorphisms that interact in a complex fashion to produce the autoimmune phenotype.
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This thesis is dedicated to the memory of my beloved mother-in-law Ms. Kitty Chui
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LIST OF ABBREVIATIONS

Abs – antibodies
AICD – activation induced cell death
ANAs – anti-nuclear antibodies
APCs – antigen presenting cells
Ars – p-azophenylarsonate
BAFF – B-cell activating factor
BANK1 – B-cell scaffold protein with ankyrin repeats 1
BCR – B-cell receptor
BI – beef-insulin
BLK – B cell lymphocyte kinase
BLys – B lymphocyte stimulator
Cr2 – complement receptor 2
CRP – C reactive protein
DAF – decay accelerating factor
DCs – dendritic cells
FDCs – follicular dendritic cells
cDCs – conventional dendritic cells
mDCs – myeloid dendritic cells
pDCs – plasmacytoid dendritic cells
DILE – drug induced lupus erythematosus
dsDNA – double stranded DNA
ssDNA – single stranded DNA
EBNA-1 – Epstein-Barr nuclear antigen-1 protein
EBV – Epstein-Barr virus
ENU – alkylating agent ethylNitrosourea
FTOC – fetal thymic organ culture
α-GalCer – α-galactosylceramide
GCs – germinal centres
GM-CSF – Granulocyte-Macrophage Colony Stimulating Factor
GN – glomerulonephritis
GVHD – graft-versus-host disease
GWAS – genome wide association studies
HEL – hen egg white lysozyme
sHEL – soluble hen egg white lysozyme
HEVs – high endothelia venules
IFN-α - interferon alpha
Ig – immunoglobulin
IL – interleukin
IRAK1 – IL-1 receptor-associated kinase-1
IRF5 – IFN regulatory factor 5
ITAMs – immunoreceptor tyrosine activation motifs
ITIMs – immunoreceptor tyrosine inhibitory motifs
ITGAM – Integrin alpha M
LOD score – logarithm of the odds score
LPS – lipopolysaccharide
LY9 – lymphocyte antigen 9
LYN – V-yes-1 Yamaguchi sarcoma virus related oncogene homolog
MASP – marker assisted selection protocol
M-CSF – macrophage colony stimulating factor
MCP-1 – monocyte chemoattractant protein-1
MECP2 – methyl CpG binding protein 2
MHC – major histocompatibility complex
MIF – macrophage migration inhibitory factor
Mls – minor lymphocyte stimulating antigens
NKT – natural killer T cells
NSAIDs – non-steroidal anti-inflammatory drugs
PDCD1 – programmed cell death -1
PTPN22 – protein tyrosine phosphatase non-receptor type 22
RA – rheumatoid arthritis
RAG-1 – recombination activating gene 1
RAG-2 – recombination activating gene 2
RBC – red blood cell
mRNA – messenger RNA
SAP – SLAM associated protein
SAPK – stress protein activated kinase
SCID – severe combined immunodeficiency
SHP-1 – Src homology region 2 domain-containing phosphatase-1
SHIP-1 – Src homology 2-domain-containing inositol polyphosphate 5’-phosphatase
SLAM – signaling lymphocyte activating molecule
SLE – systemic lupus erythematosus
SNP – single nucleotide polymorphism
SPP1 – secreted phosphoprotein 1
SSR – simple sequence repeats
STAT4 – signal transducer and activator of transcription-4
TCR – T-cell receptor
mTEC – medullary thymic epithelial cells
Tg – transgene/transgenic
dTg – double transgenic
T_{FH} – T follicular helper cells
T_{h1} – T helper 1 cells
T_{h2} – T helper 2 cells
Tr1 – T regulatory type 1 cells
TLRs – Toll-like receptors
TNF-α – tumour necrosis factor alpha
TNFAIP3 – TNFα-induced protein-3
TNFSF4 – TNF superfamily gene 4
TREX1 – three prime repair exonuclease 1
UTR – untranslated region
UV – ultra violet
Yaa – Y-linked autoimmune accelerator
LIST OF EXPERIMENTAL MOUSE STRAINS DESCRIBED IN
THESIS

B6 – C57BL/6 mouse
B6.dTg – C57BL/6 mouse with anti-HEL Ig and sHEL transgenes
B6. Ig Tg – C57BL/6 mouse with the anti-HEL Ig transgene
B6.TCRα-/− – C57BL/6 mouse with a TCRα gene deletion
B6.Thy1αIgHα – C57BL/6 congenic mouse with Thy1α and IgHα alleles
B6.NZBc1(35-106) – C57BL/6 congenic mouse with a NZB 35-106 cM chromosome 1 interval
B6.NZBc1(82-106) – C57BL/6 congenic mouse with a NZB 82-106 cM chromosome 1 interval
B6.NZBc1 – another term for B6.NZBc1(35-106) mice
B6.NZBc1L – used to refer to B6.NZBc1(35-106) mice in some studies
B6.NZBc1L.dTg – B6.NZBc1(35-106) mouse with anti-HEL Ig and sHEL transgenes
B6.NZBc1L.TCRα-/− – B6.NZBc1(35-106) mouse with a TCRα gene deletion
B6.NZBc1S – used to refer to B6.NZBc1(82-106) mice in some studies
B6.NZBc1(43-85) - C57BL/6 congenic mouse with a NZB 43-85 cM chromosome 1 interval
B6.NZBc1(96-100) - C57BL/6 congenic mouse with a NZB 96-100 cM chromosome 1 interval
B6.NZBc1(96-100).dTg – B6.NZBc1(96-100) mouse with anti-HEL Ig and sHEL transgenes
B6.NZBc1(88-100) – C57BL/6 congenic mouse with a NZB 88-100 cM chromosome 1 interval

B6.NZBc1(70-100) – C57BL/6 congenic mouse with a NZB 70-100 cM chromosome 1 interval

B6.NZBc1(35-102) – C57BL/6 congenic mouse with a NZB 35-102 cM chromosome 1 interval

B6.NZBc13(24-73) – C57BL/6 congenic mouse with a NZB 24-73 cM chromosome 13 interval

B6.NZBc13 – another term for B6.NZBc13(24-73) mice

B6.NZBc1c13 – C57BL/6 congenic mouse with both NZB 35-106 cM chromosome 1 and NZB 24-73 cM chromosome 13 intervals
CHAPTER 1

INTRODUCTION

Portions of this chapter have been adapted from Seminars in Immunology, 2009, 21(6): 372-82.
1.1 HUMAN LUPUS PATHOGENESIS

Systemic lupus erythematosus (SLE) is a severe chronic multisystem autoimmune disease of unknown etiology characterized by the loss of tolerance to a variety of self-antigens, including cell membrane, cytoplasmic, phospholipid-associated, nervous system, and most importantly nuclear antigens, such as chromatin and dsDNA (double stranded DNA) (1). This loss of tolerance leads to production of a wide variety of autoantibodies (ANAs) directed against nuclear antigens including anti-chromatin, -histone, -ssDNA, -dsDNA, -Ro, -La, -Sm, and –snRNP antibodies, that result in the formation of immune complexes (reviewed in (2) and (3)). Deposition of immune complexes in the glomeruli, skin, joints, and other organs induces tissue damage resulting in the manifestations of disease which include glomerulonephritis, skin rashes and arthritis (4-6). SLE is a chronic remitting and relapsing disease associated with a significant morbidity and mortality. Notably, about 70% of SLE patients develop immune complex mediated glomerulonephritis among which approximately 40% of the cases result in severe kidney damage.

The worldwide prevalence of SLE is highly variable but conservatively estimated at 19–241 cases per 100,000 individuals, depending on the geographical area, ethnicity and the methods of ascertainment (reviewed in (7)). There is a strong gender imbalance in the prevalence of SLE as it mainly affects women of childbearing age, with the highest incidence of disease occurring between the ages of 14 to 44 in the United States (8,9). It is estimated that the ratio of occurrence in females to males ranges from 4:1 in the pediatric population (10) to 14:1 in the adult population (11) with an average ratio of 9:1 (8). Multiple studies have demonstrated the importance of hormones such as estrogen as
a predisposing factor for the increased prevalence of SLE among females (12,13). Post-menopausal women are less likely to develop lupus (14) and post-menopausal estrogen replacement therapies as well as the use of oral contraceptive pills have been shown to exacerbate the severity of lupus (15,16). In men with Klinefelter’s syndrome (47, XXY), a common (~17 out of 10000) sex chromosomal disorder (17), there is an increased risk of developing lupus which is similar to women of the same ethnicity (18). This suggests that there is a possible gene dosage effect from the X chromosome in determining the risk of SLE. Consequently, understanding the genetic polymorphisms on the X chromosome that promote lupus might provide important clues into the etiology of SLE.

Genetic susceptibility plays an important role in the pathogenesis of human SLE. In SLE there is an approximately 24 to 70% concordance rate between monozygotic twins compared with a 2 to 5% rate in dizygotic twins. Furthermore, the risk of an individual developing SLE who has a sibling with the disease is increased >20 fold over that of the general population (λs > 20). Familial clustering is also seen among the first degree relatives of SLE patients (19-22). It is interesting to note that there is also an increased risk among families of SLE patients for the development of other autoimmune problems including rheumatoid arthritis. This finding suggests that the same genetic polymorphisms could predispose to multiple autoimmune diseases (20,23).

There is high variability in the SLE incidence rate between different ethnic populations. According to a 1994 study in the United Kingdom, women of African-Caribbean descent have the highest likelihood of developing SLE (207 per 100,000) whereas females of Asian (48 per 100,000) and Caucasian (20.3 per 100,000) ancestry are less likely to develop SLE (24). The estimated prevalence of SLE among European
women is 50-75% lower than non-European descent (23,25). Furthermore, a recent study compared the rate of occurrence of SLE with the relative genetic contribution of West African (high risk) or European (low risk) ancestry in the lupus patients’ genome. The results supported an additive susceptibility model as an explanation for the differences in the likelihood of developing lupus between ethnic groups and demonstrated that the risk of lupus is associated with the number of copies of high risk alleles at the disease loci, which are more commonly found in West Africans (26).

1.2 DIAGNOSIS AND TREATMENT

Owing to the diverse symptoms and clinical manifestations associated with SLE over the time course of the disease, diagnostic indices have been developed to assist with diagnosis. Currently, the American College of Rheumatology (ACR) classification criteria for SLE is the most widely used (4,27,28). Based on the system developed by the 1982 Diagnostic and Therapeutic Criteria Committee of the American College of Rheumatology, a patient is diagnosed with SLE when an individual displays at least four of the 11 established criteria including malaria rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal disorder, neurologic disorder, hematologic disorder, immunologic disorder and the presence of antinuclear antibodies (28). These criteria have been validated in multiple studies to accurately diagnose SLE and to discriminate it from other conditions.

There is currently no effective curative treatment for patients diagnosed with SLE. However, multiple treatment strategies have been developed to relieve the symptoms and prevent flares in SLE patients. Anti-malarial drugs such as hydroxychloroquine (29) and
non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, naproxen and piroxicam have been effective in treating mild clinical symptoms. More powerful drugs including prednisone and cytotoxic agents, such as methotrexate, imuran, mycophenylate mofetil, and cyclophosphamide, have been used to treat the more severe life-threatening manifestations of SLE, and have resulted in significant improvements in the early mortality associated with this condition (30). The development of these new treatments has significantly improved the 5 year survival rate post diagnosis from 50% in the 1950s to above 90% (31).

Recently, B cell directed therapy has come into favor for treatment of SLE. These include anti-CD20 (rituximab and ocrelizumab) and anti-CD22 monoclonal antibody treatment (epratuzumab) that directly inhibit and deplete B cells (32). Although early studies of these agents showed some promise, recent trials have been disappointing. In addition to depleting B cells, blocking the cytokines required for the survival and differentiation of B cells has been used for treatment of human SLE. Belimumab, an anti-BAFF antibody that blocks BAFF function, has been shown to reduce the autoantibody levels as well as B cell counts in SLE patients and to date is the only biologic therapy to demonstrate efficacy in a randomized clinical trial of SLE patients (33). Treating patients with ruplizumab, a humanized anti-CD40L monoclonal antibody, also initially demonstrated some effectiveness in reducing the severity of SLE. However, this drug was dropped from testing as a result of serious life-threatening side effects, most notably thromboembolisms (34).
1.3 THE ENVIRONMENT AND SLE

Although genetic factors play a vital role in predisposition to SLE, the lack of 100% concordance in monozygotic twins has been cited as evidence that additional factors, possibly environmental in origin contribute to the pathogenesis of SLE (35). Proposed environment factors include: drugs, chemicals, infectious exposures, and sun exposure.

There is evidence to suggest that exposure to drugs and chemicals including aromatic amines, hydrazines, silica dust, vinyl chloride, organic solvents, heavy metals or even tobacco smoke is associated with the development of SLE (36). A 1996 study by Conrad et al. found that male uranium mine workers that have been exposed to high levels of silica dust are ten times more likely to develop SLE than the general population (37). In addition, over 80 prescribed drugs have been described to cause drug induced lupus erythematosus (DILE) and the number is constantly increasing. These drugs can be divided into 10 broad categories with distinct pharmacological and therapeutic functions including: anti-arrhythmics (Procainamide), anti-hypertensives (Hydrazines), antibiotics (Minocycline) and anti-inflammatory (D-Penicillamine) medications (38). Anti-TNFα therapies, which are used for treatment for Rheumatoid Arthritis (RA), such as Infliximab and Etanercept have also been linked to DILE (39). However, DILE, unlike SLE, is a completely reversible autoimmune condition, as symptoms generally subside within weeks or months upon withdrawal of the culprit drug.

A number of infections have also been linked to the development of lupus, including EBV (Epstein-Barr virus), measles and rubella, as elevated level of antibodies against these viruses has been observed in lupus patients. The evidence is strongest for
EBV, where it has been demonstrated that mice injected with plasmids expressing EBNA-1 (Epstein-Barr nuclear antigen-1 protein) develop anti-dsDNA and anti-Sm autoantibodies (40). It has been shown that the viral protein EBNA-1 shares sequence homology with the autoantigen small nuclear ribonucleoprotein SmD, suggesting that EBV infection might lead to development of lupus through molecular mimicry (41).

Exposure to UV radiation is another environmental factor that can trigger the development of cutaneous and systemic lupus. In fact, sun light avoidance is one of the recommendations in management of lupus patients (42). Although the exact mechanism by which UV radiation leads to lupus remains the subject of study, it has been demonstrated that high doses of UVB (UV subtype B) radiation can induce apoptosis of keratinocytes, resulting in expression of nuclear antigens on the cell surface of apoptotic keratinocytes. These clusters of translocated cell surface nuclear antigens, together with the presence of anti-nuclear autoantibodies, form immune complexes in the circulation that could deposit in various organs and induce flares of lupus disease activity (43).

1.4 LUPUS SUSCEPTIBILITY GENES IN HUMANS

Genome wide association studies (GWAS), a hypothesis free approach that allows researchers to efficiently scan genetic polymorphisms across the genomes large populations and identify genetic variants associated with a particular disease, have become feasible following the completion of the Human Genome Project in 2003 and the availability of high-throughput single nucleotide polymorphism (SNP) genotyping technologies. Although SLE has been a relative late-comer to GWAS, four GWAS have
been completed so far resulting in the identification of novel SLE susceptibility genes as well as confirmation of several risk alleles previously found using the candidate gene approach (reviewed in (44)and (45)). The HLA region demonstrated the strongest association with human lupus in European-American population (46). Genes within the class II HLA-DR region that impact on antigen presentation as well as the class III HLA that regulate the innate immune system have been implicated in the pathogenesis of human SLE (46-48). Within this region both complement components and complement regulatory proteins have been associated with human lupus. A homozygous C1q deficiency in humans has been shown to induce the development of lupus with a high degree of penetrance (49,50) and rare risk variants of C2, C4A and C4B in human also confer increased risk for the disease (52). Genetic variations in C reactive protein (CRP), one of the acute phase plasma proteins whose level increases during systemic inflammation and facilitates the clearance of apoptotic debris, have also been associated with human lupus (51).

GWAS have also led to identification of a set of genes related to TLR and pro-inflammatory signaling that are associated with an increased risk for lupus. These include: IRAK1 (IL-1 receptor-associated kinase-1), TNFAIP3 (TNFα-induced protein-3) and MECP2 (methyl CpG binding protein 2) that are downstream of TLR signaling (53-55); IRF5 (IFN regulatory factor 5) and STAT4 (signal transducer and activator of transcription-4) that are downstream of IFNα signaling (55-57); as well as SPP1 (secreted phosphoprotein 1) and TREX1 (Three prime repair exonuclease 1) that regulate IFNα production in dendritic cells (58,59).
A number of additional genetic variants associated with development of human lupus regulate T and B cell signaling or T-B cell collaboration. Genetic polymorphisms predicted to impact predominantly on B cell signaling are \textit{FCGR2B BANK1} (B-cell scaffold protein with ankyrin repeats 1), \textit{BLK} (B cell lymphocyte kinase) and \textit{LYN} (V-yes-1 Yamaguchi sarcoma virus related oncogene homolog) are linked to an increased risk for lupus (55-57). While those predicted in impact on both T and B cell signaling or T-B collaboration include \textit{PTPN22} (protein tyrosine phosphatase non-receptor type 22), \textit{STAT4}, \textit{PDCD1} (programmed cell death -1), \textit{LY9} (lymphocyte antigen 9), and \textit{TNFSF4} (TNF superfamily gene 4) (55-57,60-63). Risk variants that promote lymphocyte survival such as the rare dominant negative \textit{FAS} variants have also been associated with human SLE (64-66).

Finally, a number of risk variants identified in GWAS as being associated with SLE are likely to predominantly affect end organ damage. Genetic variants of \textit{FCGRIIA} and \textit{FCGRIIIA} that encode the low binding isoforms of these proteins are proposed to confer an increased risk for renal disease in human lupus due to impaired clearance of immune complexes (56,67). Recently, risk variants in \textit{ITGAM} (Integrin alpha M), encoding integrin\textalpha_M (CD11b) that functions in immune complex clearance, leukocyte activation, and cell adhesion and migration of neutrophils and monocytes, were also found to be associated with human SLE, possibly through the induction of end organ, particularly renal, disease (56). Finally, similar to what was observed recently for lupus prone mice, genetic polymorphisms in the kallikrein gene family such as \textit{KLK1} and \textit{KLK3} were found to be associated with an increased risk of renal disease in human lupus (68).
1.5 MURINE MODELS OF SLE

1.5.1 Transgenic and Knockout Models of SLE

Identification of the immune abnormalities that lead to the development of SLE has been greatly aided by the study of knockout mice and transgenic mouse strains. In general, genetic modifications that promote lupus can be classified into four groups. 1) Those that promote presentation or impair clearance of apoptotic debris; 2) those that decrease the threshold for B or T cell activation; 3) those that lead to impaired apoptosis/survival of self-reactive lymphocytes; and 4) those that promote end organ damage as illustrated in Table 1.1.
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<th>Proposed mechanisms</th>
<th>Mouse</th>
<th>Candidate genes from congeneric studies</th>
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<td>Complement &amp; clearance</td>
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<td>Tir8 &lt;sup&gt;-/-&lt;/sup&gt;</td>
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### H-2

#### Aberrant lymphocyte signaling

| B cell signaling | CD22 <sup>-/-</sup> | SHP-1 <sup>me<sup>+</sup></sup> |
|                 | Lyn <sup>-/-</sup> | |
|                 | FcγRIIb <sup>-/-</sup> | FcγR2b |
|                 | CD19 Tg | CR1/CR2 |
| T cell signaling | G2A <sup>-/-</sup> | Ly108 (SLAM) |
|                 | Gadd45a <sup>-/-</sup> | Coro1a |
|                 | p21 <sup>-/-</sup> | |
|                 | Roquin <sup>smash</sup> | |
|                 | Ro52 <sup>-/-</sup> | |
|                 | CTLA-4 <sup>-/-</sup> | |
| Other | CD45 E613R | |
|       | Pdc1 <sup>-/-</sup> | |
|       | Rai <sup>-/-</sup> | |
|       | p66ShcA <sup>-/-</sup> | |

### Promote survival of autoreactive lymphocytes

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<td>Bim&lt;sup&gt;-/-&lt;/sup&gt;</td>
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<td>Bcl-2 Tg</td>
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<td>BAFF Tg</td>
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<td>PTEN&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>IL-2Rβ&lt;sup&gt;-/-&lt;/sup&gt;</td>
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### Promote end organ damage

|                | Kallikreins |

* Abbreviations: <sup>-/-</sup>, knock-out; Tg, transgenic; <sup>+/−</sup>, heterozygous
* Serum amyloid P-component; <sup>*</sup> Phosphatase and tensin homolog

**TABLE 1.1 Summary of genetic modifications (transgenic and knockouts) as well as candidate genes implicated in murine SLE. (Adapted from Y.H. Cheung et al, Semin. Imm. 2009 (69), page 373)**
1.5.1.1 Mutations that impair clearance of apoptotic debris and/or promote its
presentation to the immune system

In normal individuals apoptotic debris is rapidly cleared by the
macrophage/phagocytic system so that apoptotic cells are rarely seen, even in organs with
large numbers of cells undergoing programmed cell death (70-72). This rapid clearance
together with the secretion of TGF-β by macrophages following uptake of apoptotic cells,
ensures that the immune system does not become activated (73-76). Genetic
manipulations affecting the clearance of apoptotic debris promote the development of
lupus (Table 1.1). These include: the C1q, Mer (Mer receptor tyrosine kinase) and
Nhr1h3/Nhr1h2 (liver X receptor, LXR) gene deletions (77,78), which lead to defective
removal of apoptotic cells by macrophages; a genetic modification leading to the absence
of serum IgM that impairs clearance of apoptotic debris (79); and the mfge8 gene deletion
that leads to reduced uptake of apoptotic debris in germinal centres (80). It has been
proposed that the failure to clear early apoptotic cells leads to accumulation of later
apoptotic and necrotic cells, with release of nuclear debris which is immune-stimulatory
(73,81). In support of this concept, gene deletions of DNaseI or serum amyloid P that
impair removal of DNA or masking of chromatin that has been released from late
apoptotic/necrotic cells, respectively, promote lupus (73,82,83). More recently deletion
of Ro, a lupus auto-antigen that binds to and promotes clearance of misfolded ribosomal
RNA, has also been shown to promote lupus (84).

Uptake of nuclear antigens/debris through antigen-specific receptors on B cells or
Fc receptors on dendritic cells results in delivery of nucleic acids to endosomal
compartments containing nucleic acid-sensing toll-like receptors (TLRs), such RNA-
sensing TLR-3, -7, and -8, and DNA-sensing TLR-9. In B cells combined Ig and TLR-signaling has been shown to lead to cellular activation and differentiation to Ig-secreting plasma cells (85), whereas TLR-signaling in dendritic cells results in cellular activation, maturation, and secretion of pro-inflammatory factors (86). In TLR-7 transgenic mice, enhanced TLR signaling results in significantly increased B and dendritic cell activation and lupus-like autoimmunity (87). Similarly, mice lacking *Tir8/Sigrr* (Toll-IL-1 receptor 8), an orphan receptor known to suppress the TLR-mediated pathogen recognition by dendritic cells, also demonstrate abnormal lymphoproliferation and production of IgG anti-nuclear antibodies (88). These findings suggest that the strength of TLR signaling is under tight control and that disturbances that lead to enhanced signaling within the TLR nuclear-antigen sensing pathways promote SLE.

1.5.1.2. Genetic manipulations that decrease the threshold for B and/or T cell activation

A number of genetic modifications that alter the strength or nature of B and/or T cell signaling also lead to the development of lupus-like autoimmunity. A majority of knockouts or transgenic mice in this classification contain genetic manipulations that affect B cell signaling, demonstrating the importance of B cell defects in the pathogenesis of lupus. It has been shown that mice transgenic for *Cd19*, one of the many regulators of PI3K in B cell signaling, demonstrated a lower threshold of B cell activation and breach of B cell tolerance to dsDNA (89). Deletions of genes such as *CD22*, *SHP-1* (Src homology protein tyrosine kinase) as well as *Lyn* (V-yes-1 Yamaguchi sarcoma viral related oncogene homolog) also promote lupus in mice by lowering the threshold for B
cell activation (90-93). *Lyn* deficient mice demonstrated splenomegaly, elevated serum IgM levels, autoantibody production, and glomerulonephritis at 4 month of age (92). Although *Lyn* is a member of the Src family of kinases that is expressed mainly in B cells and monocytes and functions in positively mediating signals downstream of the BCR through regulating tyrosine phosphorylation of ITAMs (immunoreceptor tyrosine activation motifs), it also plays a negative signaling role. It functions to phosphorylate ITIMs (immunoreceptor tyrosine inhibitory motifs) on CD22, resulting in the recruitment of Src homology region 2 domain-containing phosphatase-1 (SHP-1) and SHIP phosphatases to the plasma membrane resulting in down modulation of BCR mediated signaling (93). In motheaten mice that are homozygous for recessive allelic mutations in the hematopoietic cell phosphatase, *SHP-1*, a lupus-like illness also develops (93-95). In these mice several point mutations result in alternate splicing of the *SHP-1* gene leading to a deficiency of functional SHP-1, an important negative regulator of B and T cell receptor signaling. Both homozygous motheaten and viable motheaten mice demonstrate severe combined immunodeficiency and systemic autoimmunity with production of pathogenic autoantibodies and deposition of immune complexes in various organs. It is likely that these defects promote lupus through breaching B cell anergy rather than through effects on clonal deletion or receptor editing of autoreactive B lymphocytes. Anti-hen egg white lysozyme (HEL) Ig and soluble HEL (sHEL) transgenes have been backcrossed onto the *CD19* transgenic, motheaten viable, CD22/- and Lyn/- backgrounds (93,96). In double transgenic motheaten viable mice, deletion of anti-HEL Ig Tg B cells was enhanced, consistent with the concept that mutations that enhanced signaling also promote B cell deletion and/or receptor editing. Similarly, increased IgM
downregulation on anergic lymphocytes was observed in CD19 transgenic anti-HEL/sHEL double transgenic mice. However, high levels of anti-HEL antibodies were produced in these mice, suggesting the presence of a breach in peripheral tolerance.

Mice deficient in *Fcgr2b*, a receptor expressed only on B cells that inhibits BCR mediated activation of B cells by antigens contained in immune complexes also demonstrated enhanced production of anti-nuclear antibodies, particularly when paired with other genetic modifications that promote lupus (97). However, in these mice B cell anergy appears to be intact, and autoantibody production is enhanced due to affects on plasma cell survival and/or differentiation.

Production of pathogenic IgG autoantibodies, a hallmark of SLE, is augmented by T cell help. Therefore it is not surprising that mice with deletions or manipulations of genes affecting T cell function are implicated in the pathogenesis of lupus. Mice with targeted disruptions of the gene *G2a*, an orphan G protein coupled receptor expressed mainly in lymphoid tissues, displayed a lupus-like syndrome including production of anti-nuclear antibodies, deposition of immune complexes in the glomeruli and enlargement of secondary lymphoid organs. T cells but not B cells from *G2a*−/− mice are hyperresponsive to in vitro stimulation with an altered threshold for activation (98). Similarly, *Gadd45a*−/−, *p21*−/−, or double knockout mice demonstrated increased mortality, severe glomerulonephritis and production of anti-ss/dsDNA autoantibodies (99). T cells from these mice exhibit a reduced activation threshold as both splenic and lymph node T cells hyper-proliferate in response to anti-CD3 or IL-2 stimulation *in vitro*, implicating *Gadd45* and *p21* in the regulation of T cell activation. At present the exact mechanisms by which *G2a, Gadd45a* and *p21* gene deletions lead to the development of
autoimmunity remain unclear, however it is possible that the reduced T cell activation threshold in these mice leads to abnormal activation of low affinity autoreactive T cells in the presence of self peptide. This in turn could provide help to self reactive B cells, leading to production of pathogenic autoantibodies.

Program Death 1, or PD-1, is a type I transmembrane protein containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) in its cytoplasmic tail. It is known to negatively regulate T cell signaling by recruiting SHP-1 and SHP-2 phosphatases to its cytoplasmic domain, thus controlling the balance between T cell activation and tolerance. Deletion of pcdl1, the gene encoding PD-1 on a C57BL/6 background results in the development of lupus like syndromes in these mice (100). B6.PD-1/- mice acquire a mild proliferative glomerulonephritis phenotype with significant deposition of IgG and complement in renal glomeruli. CD8+ cells from these mice demonstrated augmented proliferation and cytokine production in response to antigen stimulation. Using a transgenic mouse model carrying the 2C (a TCR recognizing H-2d) transgene on a H-2bd background that is either PD-1 sufficient or deficient, similar reductions in the number of 2C+ self reactive T cells in the periphery were seen suggesting the presence of intact thymic selection. Nevertheless, 2C+ T cells from PD-1 deficient mice exhibited abnormal activation and these mice died of graft-versus-host (GVH) like disease, indicating a defect in the maintenance of peripheral tolerance. It has been suggested that PD-1 functions to switch off autoreactive T cells in the periphery, leading to the induction of peripheral tolerance. Thus genetic manipulations of PD-1 could result in the breakdown of peripheral T cell
tolerance, resulting in the accumulation of activated self reactive T cells in the periphery that could support for autoantibody production.

A more recent study by Goodnow’s group took the approach of systematically generating mutations in mouse germline genes by feeding male C57BL/6 mice the alkylating agent ethylnitrosourea (ENU) that randomly induces single base mutations in mouse spermogonial cells every 0.5 Mb (101). Screening for progeny that developed features of autoimmunity including generation of anti-nuclear antibodies, this group discovered a previously unknown gene *roquin* (*rc3h1*) that encodes the protein Roquin (RING finger and C3H zinc finger protein 1), a highly conserved member of the RING-type E3 ubiquitin ligase protein family. Roquin<sup>san/san</sup> (termed *sanroque*) mice, carrying a M199R mutation in the Roquin protein, develop characteristics of SLE remarkably similar to human lupus such as presence of anti-dsDNA antibodies, deposition of IgG immune complexes in the kidney resulting in proliferative glomerulonephritis and hemolytic anemia. These mice also displayed splenomegaly, lymphadenopathy, overexpression of costimulatory molecules and the presence of increased number of germinal centres and T follicular helper (T<sub>FH</sub>) cells.

By reconstituting sublethally irradiated B6 mice with a mixture of bone marrow derived from Roquin sufficient and deficient backgrounds, it was demonstrated that the expansion of T<sub>FH</sub> cell subset in these mice is T cell intrinsic (102). Purified CD4<sup>+</sup> T cells isolated from the sanroque mice demonstrated enhanced responses to TCR crosslinking in vitro. When stimulated with plate bound anti-CD3, these T cells produced increased amounts of IL-5 and IFN-γ, and demonstrated enhanced upregulation of ICOS, suggesting the presence of a possible signaling defect in the CD4<sup>+</sup> T cell subset that
might lead to the accumulation of T<sub>FH</sub> cells, possibly through the abnormal upregulation of ICOS, an important costimulatory molecule required for the differentiation and survival of T<sub>FH</sub> cell subset. Notably, introduction of a SLAM associated protein (SAP) knockout onto the sanroque background abrogated the abnormal accumulation of T<sub>FH</sub> cells as well as the spontaneous formation of GCs with little effect on the proportion of T<sub>H1</sub> and T<sub>H2</sub> cell subsets, further supporting the roles of T<sub>FH</sub> in the formation of GCs.

When limiting the Icos expression in the sanroque mice by breeding these mice onto a Icos<sup>+/−</sup> background, the severity of autoimmune phenotypes, including splenomegaly, lymphadenopathy and the number of follicular helper cells and germinal centres (103). This finding suggests a critical mechanism in the regulation of lupus pathogenesis resides in the ICOS pathway and raises the possibility that this could be a potential therapeutic target in the treatment of SLE.

1.5.1.3 Genetic manipulations that lead to enhanced survival of autoreactive lymphocytes

Genetic modifications that lead to impaired apoptosis and enhanced survival of autoreactive lymphocytes can also promote lupus. Deletions or alterations of genes involved in both the pro-apoptotic and anti-apoptotic pathways, including Fas (lpr mutation), Fasl (gld mutation), Bim, Pten (phosphatase and tensin), Bcl-2 and baff (B cell activating factor belonging to the TNF family), have shown that these genes play a vital role in lupus pathogenesis, presumably by promoting survival of autoreactive lymphocytes (104-108). Mice with the recessive gld mutation develop a lupus-like phenotype characterized by early mortality, lymphadenopathy, and
hypergammaglobulinemia as well as production of anti-nuclear antibodies (104). Mice transgenic for BAFF, also called B lymphocyte stimulator (BLYS), also develop lupus (107). When the Baff transgene and Fas<sup>lpr/lpr</sup> or Bim<sup>-/-</sup> knockouts were crossed to a classic B cell anergy mouse model with anti-hen egg white lysozyme immunoglobulin (anti-HEL Ig) and soluble HEL (sHEL), anti-HEL Ig/sHEL double transgenic mice demonstrated a breach in B cell tolerance, suggesting that these genes play a role in lupus pathogenesis by promoting the inappropriate survival of anergic B cells (109-111).

1.5.1.4. Genetic manipulations that promote tissue damage in lupus

Once autoantibodies are produced and become deposited in tissues, deletion or alteration of genes regulating the inflammatory cascade can either enhance or reduce the severity of lupus related end organ damage. For instance, when C3aR, C5aR or DAF (Decay Accelerating Factor) were knocked out on MRL.Fas<sup>lpr</sup> background, these mice demonstrated exacerbation of autoimmune disease including accelerated glomerulonephritis and higher level of serum autoantibodies (112,113). On the other hand, deleting pro-inflammatory genes in the alternative complement pathway such as complement factor B (Bf) and factor D (Do) significantly reduced the severity of renal disease and mortality of the MRL.Fas<sup>lpr</sup> mice (114,115). Mice with deleted cytokine and/or chemokine genes such as M-CSF, MIF (macrophage migration inhibitory factor), MCP-1 (monocyte chemoattractant protein-1) and Ccr2 demonstrated impaired recruitment of macrophages and T cells to the kidney, resulting in attenuation of renal disease (116-119).
1.5.2 Spontaneous Arising Murine Models of SLE

Although study of genetically manipulated mice has provided insight into the nature of defects that can produce lupus, they represent an extreme case that may not be replicated in nature. Furthermore, they do not provide the capacity to identify novel genetic polymorphisms that may promote lupus through unknown mechanisms. Fortunately, there are a number of inbred mouse strains that spontaneously develop a lupus-like illness that may more accurately reflect the human disease. Of these the New Zealand (NZ), MRL, MRL.Fas$^{lp}$, and BXSB mouse strains have been most extensively studied.

1.5.2.1 NZ mice

There are several NZ strains that have been used to study lupus. These include the NZ Black (NZB), NZ White (NZW), (NZB x NZW)$F_1$ cross (NZB/W) and the NZ Mixed (NZM) mouse strain, which is a recombinant inbred strain derived by intercrossing NZB and NZW mouse strains with approximately 25% NZB and 75% NZW genetic material (120). These mouse strains, and in particular NZB, NZB/W, and NZM mice, have been used extensively in numerous studies to investigate the genetic defects and immune mechanisms that lead to the development of lupus. These mice are thought to be excellent animal models of human SLE as they display a female gender bias (~6:1 ratio) and develop lupus-like disease including production of anti-ssDNA, ---lymphocyte and -RBC or -dsDNA autoantibodies leading to hemolytic anemia and glomerulonephritis, similar to those observed in human SLE (121).
NZB mice were originally generated in Dr. Goodall’s laboratory (122). It was initially noted that these mice develop autoimmune hemolytic anemia and renal lesions. Subsequent studies demonstrated that the autoimmune disease in these mice is characterized by production of IgG anti-ssDNA, -lymphocyte, and –RBC antibodies resulting in hemolytic anemia at 6 to 8 month and mild glomerulonephritis at 12 month of age (reviewed in (121)). NZB mice also develop splenomegaly and exhibit polyclonal B cell activation in-vivo. Although NZB mice do not produce high titres of IgG anti-dsDNA antibodies or develop severe nephritis, they appear to possess most of the immunologic defects required. When the H-2<sup>d</sup> MHC locus of NZB mice is replaced by H-2<sup>bm12</sup> (as in NZB.H-2<sup>bm12</sup> congenic mice) anti-dsDNA autoantibodies are produced and rapidly progressive glomerulonephritis ensues (123).

Several groups, including our laboratory, have used mapping studies to demonstrate the presence of multiple lupus susceptibility loci in NZB mice that lead to the development of lupus nephritis in various NZB hybrid crosses. Four major genetic regions on chromosomes 1, 4, 7 and 13 of the NZB mouse model have been repeatedly mapped (124-126). These regions have been linked to the production of various types of pathogenic autoantibodies and the polyclonal B cell activation in these mice.

In our mapping study, we identified two major genetic regions on NZB chromosome 1 and 13 that were linked to polyclonal B cell activation (126). Using (B6 x NZB)<sub>F<sub>2</sub></sub> intercross mice, we showed that generation of IgG anti-ssDNA antibodies and abnormal B cell activation phenotypes such as increased expression of B7.1, B7.2, ICAM-1 and CD44 mapped to a broad region located on NZB chromosome 1 between 54 cM and 106.3 cM with two shallow but distinct LOD score peaks. The centromeric peak
(at 63 cM, maximum LOD = 3.71) was linked to the elevated ICAM-1 and CD44 expression on B cells whereas the peak at the telomeric end (at 92cM, maximum LOD = 3.79) was associated with increased proportion of B7.1+ and B7.2+ B cells, elevated serum level IgM and increased numbers of IgM ELISpots. A microsatellite marker located at 37cM on NZB chromosome 13 was also shown to be linked with the polyclonal B cell activation phenotype including upregulation of B7.1, B7.2, ICAM-1, and CD44 as well as increased numbers of IgM ELISpots (maximum LOD = 4.97). Notably, both the chromosome 1 and 13 intervals overlapped with regions previously linked to IgG anti-nuclear antibody production and glomerulonephritis in various crosses. Interestingly, no markers on NZB chromosome 4 or 7 demonstrated association with polyclonal B cell activation although elevated serum IgM levels as well as the number of IgM ELISpots were linked to the NZB chromosome 7 interval and chromosome 4 exhibited suggestive linkage to the proportion of IgM hi IgD lo B cells.

In 1963, NZB mice were bred with NZW mice to generate NZB/W mice. NZW mice are considered to be non-autoimmune, as they only produce minimal titres of anti-ssDNA autoantibodies and develop non-fatal kidney lesions. Although NZB/W mice were originally bred for coat color they demonstrated premature mortality beginning at ~6 month of age. Upon careful examination, it was revealed that these mice developed a severe immune-complex mediated glomerulonephritis beginning around the age of 5 to 7 month, resulting in fatal renal failure (127). These mice also produce high affinity IgG anti-dsDNA (whereas NZB mice produce only anti-ssDNA autoantibodies) and anti-nucleosome autoantibodies.
It has been demonstrated that the most important disease accelerating genetic contribution of the lupus resistant NZW strain when crossed with lupus prone NZB mice comes from the MHC locus located on chromosome 17 (128). Although anti-dsDNA antibody production has been implicated in the renal disease of NZB/W mice, these mice also have high serum titres of anti-gp70 antibodies, which when complexed with the viral envelop protein gp70, have been shown to lead to the formation of immune complexes that can promote development of severe glomerulonephritis (129).

By selectively intercrossing the progeny of the NZB/W mice, a total of 27 recombinant inbred New Zealand Mixed (NZM) lines were generated in the Wadsworth Centre for Laboratories Research (reviewed in (130)). Each NZM strain has a unique combination of the NZB and NZW genome. Even though almost all of the NZM lines generate anti-dsDNA autoantibodies, only some NZM strains showed an early onset of lupus nephritis with a gender bias towards females similar to the NZB/W mice. Of the 27 NZM lines generated, NZM2410 mice have been the most extensively studied, largely because this strain has been used to identify three major lupus susceptibility loci, Sle1, Sle2 and Sle3 on chromosomes 1, 4 and 7 respectively, via linkage analyses (131). Both male and female NZM2410 mice demonstrate high titres of anti-nuclear antibodies with an approximately 85% penetrance rate and develop severe lupus nephritis resulting in 50% mortality at 5 to 6 month of age.

Interestingly, studies of the NZM2410 strain have demonstrated that the majority of lupus susceptibility loci that predispose to autoimmunity were contributed by the non-autoimmune NZW background (132). In NZW mice autoimmunity is prevented by the presence of several suppressor alleles that inhibit the effects of these susceptibility loci.
The New Zealand mouse models have also been extensively crossed with other lupus resistant strains in order to identify the genetic contributions to lupus pathogenesis by both lupus prone and non-autoimmune mouse strains. One of the most well characterized F\(_1\) hybrid models is the SNF\(_1\) strain, generated by crossing NZB with lupus resistant SWR mice. These mice develop lupus like phenotypes including early mortality production of pathogenic anti-dsDNA antibodies and severe glomerulonephritis with a female bias, which are clinically similar to NZB/W mice (133-135).

### 1.5.2.2. MRL mice

The lupus-prone MRL model was derived by the Murphy group in the Jackson Laboratory in the 1960s by crossing a few standard inbred strains, including LG/J, AKR/J, C3H/Di and C57BL/6J strains. This mouse strain is one of the most extensively studied lupus prone strains (reviewed in (136)). Several lupus susceptibility loci have been mapped to chromosomes 4 (\(Lmb1\)), 5 (\(Lmb2\)), 7 (\(Lmb3\) and \(Lrdm1\)), 10(\(Lmb4\)) and 12 (\(Lrdm2\)) of MRL mice (137,138). MRL mice develop lupus like phenotypes such as the production of anti-nuclear autoantibodies and renal disease (139). However these symptoms are less severe and manifest at a later stage of life when compared with MRL.Fas\(^{lopr}\) mice containing a recessive mutation in the \(Fas\) gene discovered during the inbreeding of a MRL/Mp strain. MRL.Fas\(^{lopr}\) mice develop massive lymphadenopathy, high titre anti-dsDNA antibodies, and an extremely aggressive form of lupus nephritis (137). Fas-mediated apoptosis is absent in mice with this \(Fas\) mutation, resulting in the accumulation double negative T cells, which represent immunologically ‘exhausted’ cells that have not been deleted following previous immune responses (140). In mixed
hematopoietic chimeric mice with a mixture of Fas sufficient and deficient cells, Fas deficient cells preferentially produce antibodies indicating that defective Fas expression in B cells also plays an important role in genesis of autoimmunity in the mice (141). Notably, when the lpr defect is bred onto the C57BL/6 background, these mice display a markedly attenuated autoimmune phenotype, consistent with the presence and importance of other lupus susceptibility in the MRL background (138).

1.5.2.3. BXSB

The BXSB mouse strain is a recombinant inbred cross between the C57BL/6J and SB/Le strains, both of which do not themselves spontaneously develop lupus (142). The most striking observation with the BXSB model is that male BXSB mice develop a more rapid and aggressive lupus nephritis as compared to female BXSB mice due to the presence of a Y-linked autoimmune accelerator (Yaa) locus. Recently, it has been demonstrated that the Yaa locus contains a translocation of the telomeric end of the X chromosome onto the Y chromosome. This region contains TLR-7, one of the single-stranded RNA-sensing TLR. The presence of the Yaa locus results in an ~ 2 fold increase in the expression of TLR-7 expression intracellularly in male mice (143). Similar to what was observed in Tlr7 transgenic mice, BXSB mice overexpressing TLR7 demonstrated increased titres of auto-antibodies directed against RNA-related autoantigens and B cells from these mice demonstrated increased proliferation against the TLR-7 ligand Imiquimod in vitro. However, a recent study has suggested that additional gene(s) located in the translocated Yaa region may be required to produce the full acceleration of systemic autoimmunity as a result of this locus (144). Furthermore, B6 mice transgenic
for the Yaa locus do not display any lupus related abnormalities, suggesting the requirement of additional genetic defects in the BXSB mice to induce lupus pathogenesis. Consistent with this supposition, apart from the Yaa locus, mapping studies indicate the presence of at least 5 lupus susceptibility loci identified in BXSB mice that are located on chromosomes 1 (bxs1-4) (145) and 13 (bxs6) as well as a suppressor locus located on chromosome 3 (bxs5) (146).

1.5.3 Identification of lupus susceptibility loci in spontaneously arising lupus prone mouse models

Multiple mapping studies in the various spontaneously arising lupus prone mouse strains have provided a number of insights into the genetic basis of lupus including: 1) Spontaneously arising lupus is a complex genetic disease in which multiple susceptibility loci must act in tandem to produce disease. These findings contrast with those in knockout and transgenic mice, where a single gene defect is often sufficient to promote severe disease, and predict that each susceptibility locus is likely to have a less dramatic effect on immune function. 2) It is the balance between lupus-susceptibility loci and suppressor loci that dictates whether disease develops. 3) There are a large number of different susceptibility loci that promote disease present on diverse chromosomes. 4) Despite the large number of susceptibility loci, the same chromosomal regions are repetitively identified as associated with lupus in multiple mouse strains, suggesting that they share susceptibility alleles.

Although a large number of functional abnormalities have been identified in spontaneously arising mouse models it has been difficult to ascribe these abnormalities to
distinct genetic loci and determine their relative contribution to the lupus phenotype due to genetic complexity in these mice. Consequently, development of congenic mice where a chromosomal region linked to the lupus-phenotype has been introgressed onto a well-defined lupus-resistant background have become a critical means of identifying specific genetic loci and their impact on immune function and the lupus phenotype. Congenic mice were first pioneered by Nobel Prize Laureate George Snell in 1948 at the Jackson Laboratory in an attempt to study tissue transplantation (147). The traditional procedure for generating congenic mice involved repeated backcrossing of the donor parental strain onto the recipient inbred strain, using markers to select for mice that carry the chromosomal region of interest in each backcross. However, this method would normally require 3 to 4 years (or 20 backcrossed generations) to complete the generation of a single congenic line and is deemed inefficient in the current research environment.

In light of this problem, several groups developed a speed congenic technique using the marker assisted selection protocol (MASP) that significantly reduced the amount of time required to generate congenic mice (reviewed in (148)). This protocol required genome wide screening of progeny using microsatellite markers after each backcross. In short, microsatellite markers, or simple sequence repeats (SSR), are used to select offspring containing the chromosomal regions of interest from the donor strain and the absence of donor genetic contaminations in other parts of the genome. This technique significantly accelerated the development of congenic strains as it reduced the time required to generate a congenic line from an average of 3 to 4 years down to 1.5 to 2 years. Furthermore, this technique allows control over the undesired issue of donor genome contamination, permitting researchers to determine whether the phenotypes
observed in congenic mice are caused by the target gene(s) located in the congenic interval instead of undesired genetic contamination from the donor.

This technique has been applied by ourselves and others to generate congenic mice with chromosomal intervals containing susceptibility loci from lupus prone mice. To date intervals from NZB, NZM, BXSB, and MRL have been used to generate congenic mice. Chromosomal intervals from chromosomes 1, 4, 7, and 13 identified in various mapping studies are shown in Figure 1.1 (131,132,137,138,149-161).
FIGURE 1.1 Genetic maps of chromosome 1, 4, 7 and 13 congeneric lines used in our laboratory. Locations (in Mb) of each lupus susceptibility locus identified in various mapping studies are shown to the left of each chromosome representation, with the name of these loci shown to the right. Genetic origins (mouse strains) of each locus are identified in the box below each chromosome representation. Genes shown in brackets are the possible candidate genes located in each locus.
1.5.3.1 Chromosome 1 Congenic Mice

The telomeric end of murine chromosome 1 in lupus prone mouse strains, including NZB, NZM, BXSB and MRL mice, has been repeatedly mapped in various linkage analyses as predisposing to the development of GN and production of anti-nuclear antibodies (131,145,153,162). Several congenic strains have been generated to dissect the contributions of susceptibility loci in this region to the development of lupus, including B6.Sle1 from NZM2410 mice (interval of NZW origin) (163), B6.NZBc1 and B6.Nba2 from NZB mice (164,165), and B6.MRLc1 from MRL mice (162).

B6.Sle1 congenic mice, generated by Wakeland’s group, carry a homozygous NZM2410 147 to 196 Mb chromosome 1 interval crossed onto a B6 background. These mice produce high titres of IgG anti-chromatin antibodies and demonstrate increased T cell proliferation and IFN-\(\gamma\) secretion in response to stimulation with nucleosome histone components in vitro, indicating a breach of tolerance to chromatin (166). Studies of hematopoietic radiation chimeras with a mixture of B6.Sle1 and B6 allotype-tagged bone marrow indicated the presence of intrinsic B and T cell defects in B6.Sle1 mice (167). Only B6.Sle1 B cells differentiated into autoantibody producing cells in these mice, suggesting the presence of B cell tolerance defect. B cell tolerance in these mice has been further explored through investigation of anti-HEL Ig transgenic mice that have been crossed with soluble or membrane HEL transgenes or by introduction of a knockin \(V_H\) heavy chain transgene that encodes “dual specificity” for hapten p-azophenylarsonate (Ars) and nuclear antigens termed HKIR. Although tolerance to membrane HEL was retained, double transgenic mice with anti-HEL Ig and sHEL transgenes produced anti-HEL antibodies, indicating a breach of B cell anergy (168). This appeared to arise from
defective anergy induction since B6.Sle1 B cells from double transgenic mice demonstrated enhanced responses to HEL in vitro (168). Germinal centre tolerance mechanisms are also defective in B6.Sle1 mice. HKIR B6.Sle1b B cells demonstrated increased germinal centre expansion and differentiation to autoantibody producing cells as compared to those from B6 mice (169).

The Sle1 locus has been further subdivided into three separate loci (Sle1a, Sle1b and Sle1c) that each independently contribute to the development of the autoimmune phenotype. Of these, Sle1b is the strongest locus with the highest penetrance for anti-nuclear antibody (ANA) production (163). Using a positional cloning approach, the highly polymorphic cluster of genes in the SLAM/CD2 family was identified as potential candidate genes in the Sle1b locus (170). Of these molecules, Ly108 has been implicated in the regulation of B cell tolerance in B6.Sle1b mice (168). Ly108 is a self-ligating membrane glycoprotein expressed by B cells, T cells, NK cells and APCs (171,172) that has two isoforms resulting from alternate splicing (172). B6 mice carry the Ly108.2 allele whereas NZM2410, 129, NZB, NZW, BXSB, and MRL mice bear the Ly108.1 allele (170). Functional differences in the two Ly108 isoforms were found in WEHI cells transfected with the Ly108.1 allele, which demonstrated reduced cell death, lower RAG expression and reduced Ca^{2+} influx upon IgM crosslinking (168). These phenotypes were similar to those observed for B6.Sle1b immature B cells, suggesting that this Ly108 polymorphism may breach B cell tolerance by promoting increased survival and impaired receptor editing of self-reactive B cells.

In contrast to the observations for immature B cells, transfection of a mouse T cell line with Ly108.1 allele resulted in stronger SAP-dependent protein tyrosine
phosphorylation of \textit{Vav-1} and \textit{c-Cbl} (173). This is consistent with the observation that \textit{Sle1b} T cells are hyper-responsive to anti-CD3 stimulation (174). Taken together, these observations suggest genetic variation in \textit{Ly108} may play an important role in the induction of lupus in B6.Sle1b mice through effects on both B and T cell function and tolerance.

\textit{B6.Sle1a} mice have increased proportions of CD4\(^+\)CD69\(^+\) recently activated and CD4\(^+\)CD44\(^+\)CD62L\(^{lo}\) memory subsets, elevated expression of ICOS on CD4\(^+\) T cells together with an accumulation of histone reactive CD4\(^+\) T cells suggesting that this locus affects T cell function (175). Using an adoptive transfer model, B6.Sle1a CD4\(^+\) T cells were capable of activating B cells from both lupus prone as well as lupus resistant mice and could provide support for production of IgG anti-chromatin autoantibodies. B6.Sle1a mice demonstrated reduced proportions of splenic CD4\(^+\)Foxp3\(^+\) regulatory T cells and B6.Sle1a CD4\(^+\)CD25\(^+\) regulatory T cells exhibited a reduced ability to suppress in vitro. Interestingly, B6.Sle1a CD4\(^+\) T cells were also more resistant to suppression by regulatory T cells in vitro and demonstrated increased proliferation and production of IL-2, IL-4 and IFN-\(\gamma\) when stimulated with a combination of anti-CD3 and anti-CD28 Ab. In a recent study the \textit{Sle1a} locus was shown to be composed of two interacting subloci, \textit{Sle1a.1} and \textit{Sle1a.2}, that are both required in the full expression of autoimmune phenotype (176).

B6.Sle1c mice also demonstrate GN and IgG anti-chromatin Ab production, but at lower penetrance than is observed for B6.Sle1b mice (163). In the \textit{Sle1c} interval, a single nucleotide polymorphism (SNP) has been identified in the \textit{Cr2} gene which has been shown to decrease the capacity of CR1/CR2 to bind C3d (177). Consistent with altered
CR1/CR2 function, B6.Sle1c B cells showed a reduced Ca\textsuperscript{2+} influx upon BCR and CR1/CR2 crosslinking. However, the impact of this defect on B cell tolerance has not been investigated (177). More recently, analysis of B6.Sle1c CD4\textsuperscript{+} cells revealed the presence of an intrinsic T cell defect leading to accumulation of activated T cells and a reduced proportion of regulatory T cells (178). Similar to B6.Sle1a congenic mice, increased proliferation and cytokine production (IL-2, IL-4 and IFN-\textgamma) by CD4\textsuperscript{+} T cells upon stimulation by anti-CD3 and anti-CD28 Ab as well as reduced proportion of CD4\textsuperscript{+}CD25\textsuperscript{+} regulatory T cells were observed in B6.Sle1c mice.

Congenic mice with NZB chromosome 1 intervals have been produced independently by our laboratory and others. B6.Nba2 congenic mice carrying a homozygous 155 to 194 Mb interval from NZB chromosome 1 produce IgG anti-chromatin antibodies and develop mild GN (164). Our laboratory generated B6.NZBc1 congenic lines with varying NZB chromosome 1 intervals, of which B6.NZBc1(82-106) mice (with a 158 to 190 Mb interval) displayed a very similar phenotype to that observed B6.Nba2 mice (165). We also generated another NZB chromosome 1 congenic mouse called B6.NZBc1(35-106) mice with a longer NZB chromosome 1 interval (63.1 to 192.1 Mb). This congenic mouse strain made significantly higher titres of IgG anti-ssDNA and –chromatin autoantibodies, and developed more severe renal disease when compared with B6.NZBc1(82-106) mice (165). These mice also exhibited more severe splenomegaly, increased number of splenocytes, and elevated proportion of recently activated and memory B and T cells.

To further examine B cell tolerance defects in the NZB chromosome 1 congenic mice, anti-HEL Ig and sHEL transgenes were bred onto the congenic mouse
backgrounds. Both B6.NZBc1(35-106) and B6.NZBc1(82-106) dTg mice demonstrated a breach of tolerance to self antigens as they produced high titres of anti-HEL antibodies. However, upon adoptive transfer of anti-HEL IgTg B cells into sHEL recipients, B6.NZBc1(35-106) B cells demonstrated improved survival, proliferation and antibody production when compared with B6.NZBc1(82-106) mice. This indicates the presence of at least two distinct loci in the 35-106 interval where the telomeric interval encodes an intrinsic B defect whereas the centromeric interval can modulate B cell survival, possibly through increased T cell help.

NZB and NZM mice share similar SLAM/CD2 gene clusters (170), so it is likely that this breach of tolerance arises in part from the Ly108 polymorphism. However, additional candidate genes within the NZB 96-100 cM have been identified that are also proposed to alter B cell function. Rozzo et al. found increased expression of Ifi202, an IFN-inducible transcriptional regulator, in B6.Nba2 mice (164). This was reported to result from a promoter polymorphism and evidence was provided that there was reduced B cell apoptosis in B6.Nba2 mice. Increased expression of p202 can inhibit p53 dependent transcriptional activation of target pro-apoptotic genes Gadd45 and p21 (179). Interestingly, both Gadd45 and p21 knockout mice develop lupus like phenotypes, raising the possibility that the Ifi202 polymorphism in NZB chromosome 1 congenic mice induces lupus through inhibition of p53 mediated apoptosis (99). However, a more recent study has suggested that Ifi202 does not induce the development of lupus in B6.Nba2 congenic mice (180). This group narrowed down the Nba2 susceptibility locus to a 6.8-Mb interval that excludes Ifi202.
**Fcgr2b** is also polymorphic in the NZB 96-100 interval, where a deletion in the promoter region is associated with reduced levels of FcγRIIB, particularly in the germinal centres (181,182). However, deletion of FcγRIIB has been shown to augment entry of B cells into the plasma cell compartment without affecting other tolerance checkpoints (183-185), suggesting that this does not contribute to the breach of anergy in B6.NZBc1(96-100) mice.

The *Fcgr2b and SLAM* polymorphisms in NZB mice may also facilitate autoimmune disease through effects on other cellular populations. Mice with chromosomal intervals containing one or both of these polymorphisms demonstrated abnormal expansion, activation and secretion of IFN-α by their plasmacytoid dendritic cells, suggesting that dendritic cell function may also be altered.

Similar to NZM and NZB chromosome 1 congenic mice, B6.MRLc1(82-100) mice develop splenomegaly, ANAs and GN (162). Sub-division of the 82-100 cM interval into smaller fragments reveals that the susceptibility locus in this interval is located in the 92-100 cM region which overlaps with *Nba2* and *Sle1* (186). Notably, B6.MRLc1(92-100) mice have reduced levels of *Fcgr2b* mRNA and FcγRIIB in the kidney and spleen (186).

Analysis of congenic mice with various introgressed BXSB chromosome 1 intervals, B10.Yaa.BxsI/4, B10.Yaa.BxsI, B10.Yaa.BxsI/2 and B10.Yaa.Bxs2/3 mice, suggests the presence of four independent genetic loci that augment disease (161). BXSB, MRL, and NZB mice have the same *Fcgr2b* polymorphism (187,188) and replacement of the *Fcgr2b* allele in BXSB mice with a B6 wild-type *Fcgr2b* drastically reduces the severity of GN in BXSB mice (189). Interestingly, the Bxs2 interval overlaps
with the 70-82 cM interval from NZB mice and is associated with increased production of anti-dsDNA antibodies, raising the possibility that NZB and BXSB mice may share a susceptibility allele in this region.

1.5.3.2 Chromosome 4 Congenic Mice

In addition to chromosome 1, numerous lupus susceptibility loci, including Sle2, Lbw2, Adaz1, Sbw2, Nba1, Mott-1, Imh1, Spm1, Nbwa2, Lmb1 and an unnamed locus on distal NZB c4 (peak linkage at D4Mit12), have been mapped to chromosome 4 of mouse models that spontaneously develop lupus such as NZB, NZW, NZM2410, NZM2328 and MRL mice (151,155,158,184,190,191).

Wakeland and colleagues generated B6.Sle2 congenic mice, a B6 congenic mouse line carrying the homozygous NZM2410 Sle2 locus. This locus contain both NZW (between 55 to 100Mb) and NZB (between 100 to 128 Mb) genetic material. Although these mice exhibited B cell hyperactivity, polyclonal activation, expansion of both splenic and peritoneal CD5+ B1a cells as well as elevated serum level of IgM, lupus-like phenotypes are not seen (192). Further studies have demonstrated that the Sle2 locus is composed of three independent loci, with Sle2a and Sle2b derived from the NZW and Sle2c derived from the NZB background. Each can independently give rise to the B cell abnormality observed in B6.Sle2 mice with the Sle2c locus having the greatest impact (193). Subsequently, using 3H9 and 56R transgenic mouse models (both are self reactive antigen receptors with low affinity for DNA), the Sle2 locus has been shown to mediate a breach of B cell tolerance through impaired receptor editing of self reactive B cells specific for nuclear antigens (194).
Experiments in our laboratory have produced another chromosome 4 congenic mouse model carrying a homozygous NZB chromosome 4 interval extending from 8 to 79 cM. These mice, termed B6.NZBc4(8-79), are very similar to B6.Sle2 mice in that they exhibit CD5⁺ B1a cell expansion (195).

In contrast to B6.Sle2 mice, an increased proportion of NKT cells, a special regulatory T cell subset capable of suppressing immune responses through secretion of Th2 cytokines, was observed in B6.NZBc4(8-79) mice. When these mice were bred with B6.NZBc1(70-100) mice, a NZB chromosome 1 congenic mouse line that develops systemic autoimmunity including fatal glomerulonephritis, the resulting bicongenic B6.NZBc1(70-100).NZBc4(7-89) mice demonstrated attenuated disease progression. Findings included a reduced severity of glomerulonephritis, reduced incidence of mortality and lower level of serum IgG autoantibodies, suggesting the presence of protective alleles in the NZB chromosome 4 interval (C. Loh et al, unpublished observation). These observations also suggest that the expanded NKT cell population might prevent the exacerbation of lupus in bicongenic mice.

Other lupus susceptibility loci located on chromosome 4 of lupus prone mouse strains have been examined using the reverse congenic mouse models where the chromosomal interval containing the genes-regions of interest is replaced by a genetic interval derived from non-autoimmune mice. Replacing the Lbw2 and Adaz1 locus from the NZB and NZM2328 lupus prone strains with either NZW or C57BL/10 intervals revealed that Lbw2 contributes to the development of glomerulonephritis and increased mortality whereas Adaz1 locus is associated with autoantibody production (196,197).
1.5.3.3 Chromosome 7 Congenic Mice

Several lupus susceptibility loci have been mapped to chromosome 7, including \textit{Sle3/5} from NZM2410 mice (of NZW origin), \textit{Nba}3 from NZB mice, and \textit{Lmb}3 from MRL mice, that are linked to development of GN and anti-dsDNA antibody production (131,138,149).

\textit{B6.Sle3} mice demonstrate accumulation of activated T cells, reduced activation induced-T cell death, and hyper-responsiveness of T cells to anti-CD3 stimulation \textit{in vitro}, in the absence of high titre ANA production (131,198,198). In bicongenic mice with \textit{Sle1} and \textit{Sle3} intervals, the \textit{Sle3} locus has been shown to augment the production of nephritogenic autoantibodies, presumably through epitope spreading (199,200). Subsequently, it was discovered that \textit{Sle3} is comprised of two independent loci termed \textit{Sle3} (between 51 to 124 Mb) and \textit{Sle5} (between 11 to 22 Mb) where each locus can independently interact with \textit{Sle1} to exacerbate GN and T and B cell activation, but variably augmented IgG ANA (\textit{Sle3} > \textit{Sle5}) production (201,202). Study of mixed hematopoietic radiation chimeric mice with a mixture of B6.\textit{Sle3} and allotype-tagged B6 cells, indicated that the T cell phenotype observed in B6.\textit{Sle3} mice does not result from a cell intrinsic defect but instead is due to increased activation by functionally abnormal dendritic cells (201,203). Both bone marrow- and spleen-derived B6.\textit{Sle3} dendritic cells demonstrate increased activation and an altered cytokine production profile. When \textit{Sle3} dendritic cells were cultured with ovalbumin-specific OT-II transgenic T cells \textit{in vitro}, these cells induced increased ovalbumin-specific CD4\(^+\) T cell proliferation of B6 and B6.\textit{Sle3} OT-II T cells as compared to B6 dendritic cells. Furthermore, adoptive transfer of these \textit{Sle3} dendritic cells into B6 recipients led to the production of IgG anti-ssDNA
and -dsDNA antibodies (203), directly implicating the B6.Sle3 dendritic cell abnormality mice in the breach of tolerance to self antigens. Introduction of an IRAK-1 gene deletion onto the B6.Sle3 background, resulted in significant reduction in IgG and IgM anti-ssDNA and -dsDNA antibody production, GN, and most importantly, decreased activation of and cytokine production by B6.Sle3 dendritic cells (54). IRAK-1 is a serine/threonine protein kinase involved in IL-1 and TLR signaling, suggesting that the altered dendritic cell function in these mice arises from defects in this pathway.

Recent studies suggest that genetic variations in the levels of kallikreins, a group of serine proteases, may contribute to the severity of GN in B6.Sle3 and B6.Sle1.Sle3 mice, by modulating the renal response to injury (68). B6.Sle3 mice express lower levels of kallikreins in the kidney, including Klk1, Klk1b3, Klk1b5, Klk1b26 and Klk1b27, when challenged with anti-glomerular basement membrane antibody. Furthermore, injection of an adenovirus expressing the Klk1 gene into B6.Sle3 mice significantly reduced the severity of kidney disease in this model (204), illustrating the protective roles of kallikreins in antibody-mediated lupus nephritis. Sequencing of the kallikrein gene cluster revealed the presence of extensive polymorphisms in the promoter region of the Klk gene cluster (Klk1, Klk1b3, Klk1b5 and Klk1b26) which may contribute to the development of this autoimmune phenotype (68).

Similar to NZM2410 mice, MRL.Fas<sup>lpr</sup> mice have a lupus susceptibility locus located on the centromeric region of chromosome 7, Lmb3 (138). The role of this locus in the production of autoimmunity has been examined by breeding the Lmb3 interval from B6.Fas<sup>lpr</sup> onto the MRL.Fas<sup>lpr</sup> background to generate MRL.B6-Lmb3.Fas<sup>lpr</sup> mice. Examination of these mice demonstrated the important role of the Lmb3 locus in
enhancing Th1 CD4+ T cell proliferation, accumulation of DN T cells, and production of ANA in MRL mice (154). A recent study has identified a disease suppressing mutation in Corola in B6.Fas<sup>lpr</sup> mice. A single C to T transition at position 784 converts glutamine to a stop codon and leads to a complete abrogation of the expression of the protein Coronin-1A (205). Coronin-1A, an actin regulator that binds Arp2/3 and inhibits actin filament nucleation, has been shown to control the motility and viability of thymic T cells (206). Deletion of the Corola gene leads to a significant deficiency of peripheral T cells due to impaired migration from the thymus, suggesting that the Corola mutation in B6.Fas<sup>lpr</sup> mice inhibits lupus by reducing the availability of T cell help for autoantibody production (207,208).

1.5.3.4 Chromosome 13 Congenic Mice

Multiple studies have linked NZB, NZW and BXSB genetic loci on chromosome 13 to the production of IgG anti-dsDNA autoantibodies, GN, gp70, anti-gp70, and/or abnormal B cell activation (126,209-211). These loci include, Bxs6, Sgp3, Nba, and an unnamed locus on chromosome 13 identified by our laboratory. Currently, all but Nba, have been further investigated in congenic mouse models.

Our laboratory has generated B6 congenic mice with a fully homozygous NZB chromosome 13 interval that we previously linked to abnormal B cell activation (denoted, B6.NZBc13(24-73cM)) (126,212). This mouse produces high titre IgM and IgG anti-chromatin and -Sm/RNP antibodies and recapitulates to some extent many of the previously mapped altered B cell phenotypes in NZB mice. These mice also demonstrated increased T cell activation and moderate myeloid dendritic cell expansion.
Using hematopoietic radiation chimeras with a mixture of B6 allotype-tagged and B6 or B6.NZBc13 bone marrow, we found that congenic B cells displayed enhanced B cell activation, suggesting an intrinsic B cell defect (C. Loh et al., manuscript in preparation). The central role of this B cell defect in the generation of abnormal cellular phenotypes and autoimmunity in B6.NZBc13 mice was demonstrated by abrogation of these phenotypes when the B cell repertoire was replaced with a non-self-reactive Ig transgene. To determine the impact of the B cell defect on B cell anergy, anti-HEL Ig and sHEL transgenes were crossed onto the B6.NZBc13 background. Although no breach of B cell anergy was seen, increased proportions of B cells expressing edited and endogenous heavy chains were observed, suggesting altered peripheral B cell selection in these mice. Thus, an intrinsic B cell defect plays an important role in initiating and maintaining the autoimmune phenotype of B6.NZBc13 mice (Loh C. et al., manuscript in preparation).

Examination of mixed hematopoietic chimeric mice with a mixture of B6.NZBc13 and B6 allotype-tagged bone marrow revealed an attenuated lupus phenotype and equivalent congenic and B6 dendritic cell expansion. These findings together with the nature of autoantibodies produced raised the possibility of an apoptosis clearance defect in B6.NZBc13 mice. To address this possibility, B6.NZBc13 mice were injected intraperitoneally with labeled apoptotic thymocytes and, consistent with impaired clearance, peritoneal macrophage uptake of these cells was reduced (Pau E. et al. unpublished observations). Similar results were observed using bone marrow-derived macrophages.

NZB/W, MRL-Fas$^{lp}$, and BXSB/Yaa mice have been shown to produce antibodies against an endogenous retroviral envelope protein, gp70, resulting in the
formation of gp70-anti-gp70 immune complexes (anti-gp70 IC) (213). Several observations suggested a role for anti-gp70 IC in GN including: a correlation between the levels of anti-gp70 IC and the severity of lupus nephritis in NZB/W mice; induction of kidney damage by transfer of monoclonal anti-gp70 Ab in non-lupus prone mice; and a correlation between anti-gp70 IC and GN in mapping studies (214,215). Subsequent genetic studies identified the \textit{Sgp3} locus on chromosome 13 of NZB and NZW mice, and led to the generation of B6 congenic mice with NZW or NZB \textit{Sgp3} intervals: B6.NZW.\textit{Sgp3}/1, -\textit{Sgp3}/2, and -\textit{Sgp3}/3, and B6.NZB.\textit{Sgp3} (216,217). Characterization of these mice revealed that more than one locus on chromosome 13, within NZW.\textit{Sgp3}/1, NZW.\textit{Sgp3}/2 and NZB.\textit{Sgp3}, controlled nephritogenic serum gp70 production. The NZB.\textit{Sgp3} intervals have recently been shown to control endogenous retroviral expression, as assessed by the increased levels of the provirus \textit{env} RNA (218). Endogenous retroviruses have been implicated in the generation of anti-gp70 and anti-chromatin antibodies. Injection of endogenous viruses into non-autoimmune mice results in generation of these autoantibodies which have been shown to bind the endogenous retrovirus, with anti-gp70 binding cell surface gp70 and anti-chromatin binding nuclear antigens on the surface of budding virions (219). This finding is intriguing, as TLR7, an intracellular sensor of viral ssRNA, is known to play a role in lupus pathogenesis. Interestingly, crossing the \textit{Yaa} locus (with the TLR7 gene duplication) onto B6.NZW.\textit{Sgp3}/2 mice, which have elevated titres of serum gp70, could induce high titre IgG anti-DNA and -chromatin production (216).

As with the \textit{Sgp3} locus, the \textit{Bxs6} locus was linked to increased production of gp70 and anti-gp70 IC in lupus-prone BXSB mice (211). The \textit{Bxs6} locus was crossed
onto the C57BL/10 background and confirmed to be sufficient for the elevated expression levels of gp70 and anti-gp70 antibodies (220). Furthermore, as seen in B6.NZB.Sgp3/2.Yaa mice, introduction of the Yaa locus onto B10.Bxs6 mice supported renal disease, and in some instances high titre anti-DNA antibody production (220).

1.5.3.5 Bicongenic and Tricongenic Mice

While study of congenic mouse strains with a single or small cluster of lupus susceptibility loci has provided insight into the types of immune defects and candidate genes that promote lupus in the spontaneously arising mouse models of lupus, these mice often have a milder autoimmune phenotype than the lupus prone parental strain. Furthermore, studies examining the genetic inheritance of lupus susceptibility have shown that disease penetrance and severity correlates with the number of lupus susceptibility loci in the genome. Hence, various groups including ourselves, have developed bicongenic or even tricongenic mice by intercrossing existing congenic mice to determine how the different genetic loci interact with each other to produce a more severe lupus phenotype.

In 1999, Wakeland’s group crossed their chromosome 1 congenic mice carrying the Sle1 lupus susceptibility locus with chromosome 7 congenic mice containing the Sle3/5 loci. The resulting B6.Sle1.Sle3/5 bicongenic mice exhibited a more severe autoimmune phenotype similar to the parental NZM2410 strain including higher titres of IgG anti-dsDNA and nephrophilic autoantibodies, demonstrating the presence of epistatic interactions between these lupus susceptibility loci (221). Although these bicongenic mice also develop more severe glomerulonephritis than either monocongenic mouse
strain alone, the penetrance of lupus nephritis remained relatively low (~25%) when compared with the parental NZM2410 (~75%) strain, suggesting the presence of another susceptibility locus was required (221). In contrast, B6.Sle2.Sle3 bicongenic mice had similar disease severity to that observed in the B6.Sle3 single congenic strain, indicating that disease severity does not simply reflect the number of susceptibility but complex interactions among the susceptibility loci (222).

The same group then went on to generate B6.Sle1.Sle2.Sle3 tricongenic mice with the 3 major susceptibility loci found in NZM2410 mice. Interestingly, the addition of Sle2 to the B6.Sle1.Sle3 bicongenic mice significantly increased the penetrance of lupus nephritis to a level comparable with the parental NZM2410 mouse strain (222).

Apart from intercrossing single congenic mice derived from the same parental strain, bicongenic mice have also been generated with congenic intervals derived from two different lupus prone mouse strains. Examples of this include B6.Sle1.Yaa, B6.Sle2.Yaa, and B6.Sle3.Yaa mice that were generated by breeding the BXSB Yaa congenic interval onto the respective NZM2410 congenic mice. Neither B6.Sle2.Yaa nor B6.Sle3.Yaa mice exhibited an augmented autoimmune phenotype, suggesting the absence of epistatic interactions between these loci. On the other hand, B6.Sle1.Yaa demonstrated accelerated disease progression with increased disease severity similar to what was observed in tricongenic and NZM2410 mice (222).

The generation of bicongenic mice has also assisted in the mapping and analyzing the mechanisms by which suppressor loci prevent the development of autoimmunity in lupus prone mice. For instance, Wakeland’s group has mapped Sles1, a suppressor locus derived from chromosome 17 of NZW mice, to a precise 956kb segment by generating
bicongenic mice carrying both Sle1 and Sles1 loci (223). This group has shown that Sles1 interacts with Sle1 locus and suppresses the development of autoimmunity by abrogating abnormal B and T cell activation, production of autoantibodies, and development of renal disease.

1.5.3.6 Overview of insights obtained from congenic mouse strains

The investigation of SLE using murine models has clearly demonstrated that the development of lupus is dependent on epistatic interactions among multiple genetic defects affecting several immunological pathways including survival and signaling of lymphocytes, presentation and response to apoptotic debris as well as the formation, clearance, and response to immune complexes in organs. These findings are consistent with those observed in genetically manipulated mice and suggest that the overall themes for disease indication in spontaneously arising lupus-prone mouse models, and likely in humans, are similar.
1.6 ROLE OF B CELLS IN THE PATHOGENESIS OF MURINE SLE

One of the hallmarks of SLE is the production of pathogenic antinuclear antibodies. Previous studies indicate that in normal individuals, anti-nuclear antibody production in prevented by several B cell tolerance checkpoints including clonal deletion, receptor editing, clonal anergy induction, and a lack of appropriate T cell help, depending on the specificity and affinity of the Ig receptor. Thus, the presence of autoantibodies in lupus indicates that one or more of these tolerance checkpoints is defective. There is now considerable evidence that intrinsic B cell functional abnormalities contribute to this breach of tolerance and play a vital role in the development of lupus.

1.6.1 Evidence for intrinsic B cell defects in murine lupus

Both NZB and NZB/W mice exhibit chronic polyclonal B cell activation early in life that often precedes and predicts the onset of immune complex mediated glomerulonephritis. Polyclonal B cell activation in these mice is characterized by hypersecretion of IgM and increased numbers of spontaneously proliferating cells and large activated B cells on discontinuous Percoll gradients. These activated B cells express increased levels of activation markers and costimulatory molecules such as CD69, B7.1, B7.2 and ICAM-1 (224-230). Adoptive transfer of cultured pre-B cell lines derived from the fetal livers of NZB and NZB/W mice into SCID and RAG-2 knockout recipients results in hypergammaglobulinemia of IgM, IgG2a and IgG3 (231,232), suggesting that intrinsic B cell defect(s) in NZB and NZB/W mice lead to the polyclonal B cell activation phenotype. .
Further evidence suggesting the presence of intrinsic B cell defects in lupus prone mice comes from the study of mixed hematopoietic chimeric mice. When lethally irradiated B6 recipients were reconstituted with a mixture of bone marrow cells derived from Ig heavy chain allotype marked B6 and lupus prone B6.NZBc1(35-106) or B6.Sle1 congenic mice, only B cells from the congenic mouse strains are capable of upregulating costimulatory molecules such as CD80 and CD86, entering the germinal centres and differentiating into antibody producing cells that generate pathogenic IgG autoantibodies (233,234). Similar findings were obtained for mixed hematopoietic chimeric experiments using a mixture of B6 and B6.Fas\textsuperscript{lpr}, or MRL and MRL.Fas\textsuperscript{lpr} bone marrow cells, where \textit{lpr} B cells preferentially produced IgG2a anti-ssDNA and –nuclear antibodies (235). These observations suggest that intrinsic B cells defects are a necessary requirement for the production of autoantibodies, even when appropriate T cell help is present.

1.6.2 B cell functional abnormalities in murine lupus

B cells from lupus-prone mouse strains demonstrate a variety of functional abnormalities. Resting B cells from NZB/W mice hyper-proliferate in response to stimulation by non-crosslinking anti-\(\mu\) and generate elevated amounts of IgM when stimulated by a combination of IL-4 and IL-5 (236,237). Experiments in our laboratory have demonstrated that purified resting B cells isolated from NZB and NZB/W mice exhibited altered responses, including increased proliferation, IgM hypersecretion and enhanced expression of costimulatory molecules, to various T cell-derived stimuli such as CD40 cross-linking and cytokines including IL-4 and IL-5 (237,238). These findings suggest that B cells from lupus prone mice may be hyper-responsive to various stimuli.
and this may contribute to the increased activation and/or differentiation of autoreactive B cells in these mice.

Reduced B cell apoptosis is another prominent feature of altered B cell function of lupus prone mouse models including NZB, NZB/W, and MRL mice. Experiments by ourselves and others have shown that resting B cells from NZB mice are less susceptible to apoptosis following IgM crosslinking (239,240). We have further localized this defect to transitional T1 B cells (240). T1 B cells isolated from NZB mice exhibited reduced apoptosis and abnormal upregulation of the anti-apoptotic protein Bcl-2 \textit{in vivo} or following IgM crosslinking \textit{in vitro}. Mature B cells from both MRL and MRL.Fas\textsuperscript{lpr} mice also demonstrate reduced apoptosis upon BCR crosslinking, suggesting that this defect is independent of the \textit{lpr} mutation (241). The apoptotic defect in these mouse strains may lead to enhanced survival of autoreactive B cells.

B cells from lupus-prone mice also demonstrate altered signalling responses. Both MRL.Fas\textsuperscript{lpr} and male BXSB mice exhibited elevated levels of phosphorylation of various signalling molecules including AKT/mTOR, ERK1/2, NF-κB and STAT3 (242). Meanwhile, upon BCR crosslinking, splenic B cells from 6 month old NZB mice demonstrated significantly reduced tyrosine phosphatase activity. Both SAPK (stress protein activated kinase) and SHIP-1 (Src homology 2-domain-containing inositol polyphosphate 5'-phosphatase) were abnormally underphosphorylated when compared with BALB/c controls (243). The results support the notion that signalling abnormalities leading to a decreased threshold for B cell activation promote lupus in spontaneously arising lupus prone mouse models.
1.6.3 Evidence for altered B cell tolerance mechanisms in murine lupus

1.6.3.1 Altered B cell receptor (BCR) editing

Receptor editing of immature autoreactive B cells plays an important role in the revision of B cell receptors through secondary Ig rearrangement, mostly at the light chain locus, to generate non-self-reactive Ig. This process is particularly important in the maintenance of tolerance to membrane associated antigens and dsDNA (244-246). There is evidence for both increased and decreased receptor editing in various lupus prone mouse models, suggesting that disturbances of these process can contribute to B cell tolerance abnormalities in these mice. Increased *rag2* expression, light chain editing and usage of distal J<sub>κ</sub>5 gene segment were observed in lupus prone NZB/W mice with an anti-DNA Ig heavy chain transgene (247-250). Similarly, significantly higher levels of *rag1* and *rag2* gene expression were seen in splenic mature B cells of B6.Sle3/5 congenic mice, suggesting accelerated Ig receptor editing in these mice might contribute to the development of lupus. In further support of this, unimmunized B6.Sle3/5 mice exhibited atypical IgG CDR3 structures frequently observed in autoreactive B cells (251).

In contrast, immature B cells, particularly pre-B cells in the bone marrow of NZB mice demonstrated decreased *rag1* expression and increased differentiation into IgM and IgG anti-dsDNA antibody producing cells upon LPS stimulation *in vitro*, suggesting that impaired receptor editing in NZB mice could be associated with generation of IgG anti-DNA autoantibodies (252). Similar observations were made using MRL.Fas<sup>lo</sup> mice bearing the 3-83 BCR (anti-H2K<sup>k</sup>) transgene. When compared with B10.D2 3-83 BCR transgenic mice, immature 3-83 B cells from the MRL.Fas<sup>lo</sup> background exhibited a
stronger calcium flux, reduced receptor editing and altered \textit{rag} mRNA expression when stimulated \textit{in vitro} (253).

1.6.3.2 B cell anergy defects

Several studies have revealed that B cell anergy is overcome in lupus-prone mice, however the mechanisms leading to this breach appear to vary between strains. Using a classic model of B cell anergy, anti-hen egg white lysozyme immunoglobulin (anti-HEL Ig) and soluble HEL (sHEL) transgenes (Tg) were crossed onto the NZB, NZB and NZM congenic, and MRL backgrounds. NZB anti-HEL Ig/sHEL double Tg mice produced increased amounts of IgM anti-HEL antibodies that approached those seen in B6 anti-HEL Ig Tg mice, suggesting that a breach of B cell tolerance had occurred. Increased level of BAFF mRNA and serum BAFF, a B cell activating factor implicated in the breach of B cell tolerance to self antigens, were detected in NZB dTg mice (N. Chang \textit{et al.}, manuscript submitted). When B cells from NZB dTg mice were adoptively transferred into sHEL recipients they demonstrated increased survival and aberrant recruitment into the follicular compartment (254), similar to the phenotypes observed in BAFF Tg mice. Treatment with TACI-Ig, that blocks BAFF, significantly reduced the aberrant survival of transferred cells, whereas treatment with anti-CD4 had a minimal effect (N. Chang \textit{et al.}, manuscript in preparation). Nevertheless, young NZB dTg mice given anti-CD4 for more prolonged periods of time produced significantly less anti-HEL antibody than those given PBS, suggesting that BAFF and CD4 cooperate to breach tolerance in NZB dTg mice. Notably, when NZB dTg B cells were incubated with BAFF and/or HEL (acts as a self antigen) \textit{in vitro}, increased expression of Bcl-2 and enhanced
survival was observed, further supporting the concept that both an intrinsic B cell defect and an extrinsic factors lead to the breach of B cell tolerance in this model of murine lupus.

A similar breach of B cell tolerance was detected in B6.NZBc1(96-100) and B6.Sle1 mice, bearing NZB and NZM chromosome 1 intervals, respectively. B6.NZBc1(96-100) and B6.Sle1 dTg mice produced elevated titres of IgM anti-HEL antibody as compared to B6 dTg controls (N. Chang et al, unpublished observation and (7)). B6.Sle1 dTg B cells also demonstrated increased upregulation of the costimulatory molecule CD86, enhanced proliferation, and reduced apoptosis upon IgM crosslinking, suggesting that induction of anergy was impaired in these mice (7). It was suggested that Ly108, a candidate gene proposed for the Sle1 interval, is responsible for the breach of B cell tolerance observed in these mice as overexpression of the susceptibility allele, Ly108.1, lead to impaired Ca\textsuperscript{2+} mobilization, deletion, and receptor editing in immature B cells. It is likely, that the breach of tolerance in B6.NZBc1(96-100) mice is due, at least in part, to this allele since NZB mice have the same Ly108 allele. However, the magnitude of the anti-HEL antibody production appears increased in B6.NZBc1(96-100) mice, suggesting that other genetic polymorphisms in this interval may contribute to the breach of tolerance in these mice. As outlined above there are two additional candidate genes in the NZB interval, Ifi202 and Fcgr2b, both of which have been proposed to alter B cell function.

In contrast, MRL.Fas\textsuperscript{lp} dTg B cells did not exhibit any intrinsic anergy defect. Using the adoptive transfer model, normal follicular exclusion and elimination of
autoreactive B cells were observed although B cell hyperactivity and increased differentiation into plasma cells were detected in MRL anti-HEL Ig Tg mice (255).

In contrast to conventional antigens, nuclear antigens have the ability to induce activation and proliferation of naïve non-tolerant B cells as well as their differentiation into antibody forming cells in the absence of T cell help (256-259). It has been demonstrated that responses to nuclear antigens involve antigen uptake through the BCR and sequential engagement of TLRs in the late endosomal compartment (260). In normal mice, self reactive B cells are rendered anergic and these anergic cells are refractory to TLR agonists. O’Neill and colleagues showed that normal anergic 3H9/Vκ8 and Ars/A1 B cells limit TLR activation by preventing the endocytic trafficking of BCR and TLR9 into late endosomes. On the other hand, autoreactive B cells isolated from MRL.Fas<sup>lpr</sup> mice proliferate in the presence of TLR agonists, suggesting the presence of a nuclear antigen-specific tolerance defect in these mice that could act to promote development of lupus (261). It is unknown whether the anergy defects in other lupus prone mouse models also impact on the ability of nuclear antigens to stimulate anergic B cells.

Additional evidence for altered Toll like receptor B cell signaling in murine lupus comes from the study of male BXSB mice, where the Yaa locus has been shown to lead to increased levels of, and responses to, TLR7. Resting B cells from male BXSB mice are hyperresponsive to anti-IgM and CD40L crosslinking and it has been demonstrated that this hyperresponsiveness is linked to the presence of the Yaa locus (262). Tlr7 transgenic mice developed lupus like phenotypes including production of anti-nuclear antibodies and IgG autoantibodies directed against RNA-associated antigens in vivo, glomerulonephritis, diminished marginal zone population, increased B cell activation and
proliferative responses to Imiquimod, a TLR7 specific ligand (87). On the other hand, TLR7 deficient lupus prone mice exhibited attenuated systemic autoimmunity such as diminished level of IgG autoantibodies to RNA-associated antigens, reduced B cell activation as well attenuated renal disease (263). These observations suggest that altered expression of TLR can lead to abnormal B cell activation and proliferative responses to stimuli resulting in exacerbation of lupus pathogenesis.

1.6.3.3. Impaired Germinal Centre Tolerance Mechanisms

Germinal centre exclusion of B cells represents another important B cell tolerance checkpoint that can prevent the development of self reactive B cells that have undergone receptor editing or somatic hyper-mutation.

Severe IgG immune complex mediated glomerulonephritis, abnormal formation of germinal centres, elevated serum level of total IgG as well as pathogenic IgG anti-DNA, -Sm/RNP and –RBC antibodies are the common features of both NZB and NZB/W mice. It was suggested that aberrant downregulation of FcγRIIB1, an important negative feedback regulator of B cell selection, in NZB and NZB/W germinal centre B cells promote development of lupus by allowing the expansion of self reactive IgG⁺ B cells and differentiation into plasma cells. At least two polymorphisms were detected in the promoter region of the NZB FcγRIIB1 allele that act to downregulate the expression of FcγRIIB1 in germinal centre B cells. However, all these autoimmune phenotypes were completely abrogated in NZB.CD40L⁻/⁻ mice, showing the importance of T-B collaborations in the germinal centres. Furthermore, as mentioned previously, B6.Sle1b congenic mice exhibited defective germinal centre tolerance mechanisms as HKIR
B6.Sle1b B cells demonstrated increased germinal centre expansion and differentiation to autoantibody producing cells as compared to B6 controls (169). These observations suggest that defect germinal centre tolerance was at least partially responsible for lupus pathogenesis in these lupus prone mice.

1.7 ROLE OF T CELLS IN THE PATHOGENESIS OF MURINE SLE

Although B cells have long been acknowledged as a major player in the pathogenesis of SLE, aberrant selection of and/or activation self reactive T cells likely also plays a role. Experiments performed in our laboratory and those of others have indicated the importance of CD4+ T cells and CD40/CD40L interactions in regulating the production of high affinity pathogenic IgG autoantibodies in NZB and NZB/W mice (230,264-266). Treatment of NZB/W mice prior to onset of disease with anti-CD4 or anti-CD40L antibody results in amelioration of lupus nephritis, reduced production of anti-dsDNA antibodies as well as an improved survival rate (265). Another study illustrated that combined treatment with CTLA-4 Ig and cyclophosphamide is more effective in prolonging survival and reducing proteinuria in NZB/W mice when compared with treating these mice with either agent alone (267). The function of CTLA-4 Ig was to block the T cell dependent response by binding to B7 molecules and disrupting CD28 mediated T cell costimulation. The results of these treatments indicate the involvement of T cells in generation of pathogenic autoantibodies and development of renal failure in some lupus prone mouse strains. In further support of this concept, NZB.CD4/- mice exhibited delayed and reduced production of anti-erythrocyte and anti-ssDNA autoantibodies (268). Nevertheless, NZB.CD4/- mice displayed abnormal
thymic architecture and increased number of splenic IgM secreting cells similar to NZB mice, suggesting that some of the immune defects in these mice are T cell-independent. We have observed similar findings in NZB.CD40L-/- mice, where production of IgG autoantibodies, hemolytic anemia, and renal disease were abrogated, but polyclonal B cells activation was unaffected.

However, contradictory results have been obtained from the study of MRL.Fas\textsuperscript{lp} mice, where the phenotype was only partially T cell-dependent. In these mice, introduction of a CD28 knockout diminished production of IgG autoantibodies and reduced the severity of lupus nephritis but had little effect on the serum levels of IgM and IgM rheumatoid factors. Similarly, MRL.Fas\textsuperscript{lp} mice lacking CD40L demonstrated reduced glomerulonephritis but continued to have significant levels of isotype switched IgG2a and IgG2b as well as IgG anti-snRNP autoantibodies (269,270).

While the above studies indicate a role for T cells in lupus, particularly in pathogenic autoantibody production, they do not address whether intrinsic T cell functional defects are required. This question has been best examined in congenic mouse models through the generation of mixed hematopoietic chimeric mice. When lethally irradiated B6 recipients were reconstituted with B6.Sle\textsuperscript{l} and allotype-tagged B6 bone marrow cells, only Sle\textsuperscript{l} expressing T cells were abnormally activated post reconstitution (234). Use of B6.Sle\textsuperscript{l},TCR\alpha/- instead of B6.Sle\textsuperscript{l} bone marrow cells in this reconstitution, resulted in reduced production of IgG antinuclear antibodies, demonstrating that the intrinsic T cell defect in B6.Sle\textsuperscript{l} mice promotes lupus pathogenesis.
Although Th1 self-reactive T cells have been identified in several murine models of lupus and are thought to provide support for autoantibody production, increasing data suggests that other T cell subsets may also be involved in the pathogenesis in lupus. As outlined earlier T_{FH} cells have been shown to play an important role in the generation of autoimmunity in the sanroque mice. These cells secrete IL-21, which has multiple immune effects that amplify autoantibody production one of which is to further augment T_{FH} generation. Interestingly, increased IL-21 mRNA and protein levels were observed in lupus prone male BXSB mice (271) and deletion of the IL-21 receptor ameliorated development of pathogenic autoantibody production and severe glomerulonephritis leading to improved survival in these mice (272). Consistent with the role of IL-21 in the expansion of T_{FH} cells, these knockout mice also had fewer T_{FH} cells. These observations implicate T_{FH} and/or IL-21 in the genesis of disease and suggest that the findings from sanroque mice can be generalized to spontaneously arising lupus. In further support of this concept increased numbers of T_{FH} have recently been identified in the peripheral blood of SLE patients.

T helper 17 (Th17) cells have also recently been implicated in the pathogenesis of lupus. These cells are developmentally distinctive from Th1 and Th2 cells as they differentiate from naïve CD4^{+} T cells in the presence of IL-6 and TGF-β (273). IL-17, a signature cytokine produced by Th17 cells, is a potent proinflammatory cytokine involved in the early immune response to extracellular bacteria and fungi. Recently, studies have shown that IL-17 producing cells play a pivotal role in lupus pathogenesis. Glomerular infiltration of Th17 cells was found in lupus prone SNF1 mice and splenocytes from these mice produced significantly elevated levels of IL-17 when
stimulated with nucleosomes (274). Remarkably, these lupus-like phenotypes, including the glomerular infiltration of Th17 cells, could be reversed by treating the mice with low doses of a histone-derived peptides that induce tolerance. Increased serum level of IL-17 and an increased proportion of splenic Th17 cells were also detected in BXD2 mice, a recombinant inbred strain derived from intercrossing C57BL/6 and DBA/2 mice. BXD2 mice exhibited increased production of IgG anti-DNA antibodies and rheumatoid factors, elevated number of germinal centres, and progressed to development of glomerulonephritis at 6 month of age as a result of deposition of immune complexes and infiltration of B and T cells in the glomeruli (275). Genetic manipulations of the IL-17 receptor in the BXD2 mice drastically reduced the number of germinal centres in the spleen and ameliorated the production of pathogenic IgG autoantibodies. While these studies that suggest that IL-17 production may be important in the immunopathogenesis of severe lupus, the precise immune mechanisms leading to the increased generation of these cells remain to be identified.

1.7.1 Altered T cell central tolerance/thymic selection

T cells from unprimed NZB, NZB/W, MRL.Fas<sup>Fas<sub>lpr</sub></sup> and/or BXSB mice have been shown to proliferate and generate cytokine in response to a range of self antigens such as U1-70K ribonucleoprotein, erythrocyte band-3, histone derived peptide, Sm-D and anti-DNA antibody derived peptide (276-280). When NZB/W mice were immunized with SmD1(83-119) peptide, a major T cell epitope of SmD1, elevated levels of pathogenic IgG anti-dsDNA and exacerbation of lupus were observed (278). However, attempts to study central tolerance defects have failed to identify any abnormality leading to
abnormal T cell development in lupus prone mice. In one study that examined clonal elimination of self reactive T cells in (NZB x SWR) F₁ and NZB/W mice, T cells bearing Vβ17a receptors (reactive to MHC class II molecules such as I-E) or Vβ8.1 receptors (reactive to Mls1<sup>a</sup>) were normally deleted in the lymph nodes and spleen (281). Nevertheless, it is possible that examination of T cell clonal deletion using superantigens and other high affinity antigens fails to detect subtle tolerance defects.

Several thymic defects have been identified in NZB and NZB/W mice. Bone marrow T cell precursors from NZB mice demonstrate a reduced capability to differentiate in fetal thymic organ culture (FTOC) and the thymic microenvironment in NZB mice exhibits an impaired ability to support T cell maturation (282). NZB mice also have reduced numbers of medullary thymic epithelial cells (mTEC), a specialized cell type that plays an important role in negative selection of thymocytes. The number of mTEC expressing the autoimmune regulator gene Aire, that encodes the protein that serves to regulate negative selection of self reactive cells in the thymus, is also reduced in NZB mice (283). Aire-deficient mice exhibit a variety of autoimmune phenotypes including increased production of autoantibodies, T cell hyperreactivity, as well as lymphocyte infiltration in multiple organs (284), raising the possibility that reduced Aire expression in NZB mice leads to impaired negative selection.

Interestingly, altered NF-κB2 signaling, elevated level of chemokines CCL19 and CCL21, as well as reduced levels of RelB expression were observed in the NZB thymus. Both CCL19 and CCL21 play important roles in the regulation of lymphoid cell trafficking as well as the organization of lymphoid organ structures (283). It has been suggested that the increased levels of CCL19 and CCL21 in the NZB thymus lead to
enhanced the intrathymic retention of mature T and B cells that has been observed in the thymus of these mice. In further support of a role for these signaling abnormalities in NZB mice, RelB deficient mice have been shown to have reduced proportions of thymic CD4^+CD25^+ regulatory T cells, a phenotype that is also observed in the NZB thymus. Finally, NF-κb2 deficient mice exhibit impaired central tolerance (285). Notably, Aire has been shown to regulate NF-κb2 signaling, raising the possibility that the altered expression of NF-κB2 in the NZB thymus is related to the altered Aire expression (286). Taken together these findings suggest that, despite the absence of impaired deletion of superantigen-reactive cells, negative selection may be altered in NZB mice.

1.7.2 Altered Peripheral T cell tolerance defect

Even in the setting of intact central tolerance mechanisms, studies indicate that autoreactive T cells can still reach the periphery. These autoreactive T cells are kept in check by the following mechanisms: (1) T cell anergy that inactivates the self reactive T cells; (2) Fas-mediated apoptosis or activation induced cell death (AICD) that removes aberrantly activated T cells from the periphery; or (3) Active regulatory mechanisms that prevent activation of the self reactive T cells that support the production of autoantibodies.

1.7.2.1 T cell anergy and deletion

T cell anergy represents a state of T cell unresponsiveness that occurs when T cells encounter antigens in the absence of co-simulation. To assess this mechanism of
tolerance in lupus-prone mice, our laboratory generated NZB and NZB/W mice carrying a beef-insulin (BI) transgene (287,288). BI is expressed at extremely low levels in the serum and previous work has demonstrated that the major mechanism of tolerance in the BI transgenic model is clonal anergy induction. Interestingly, both NZB and NZB/W BI-transgenic mice did not spontaneously produce BI-specific antibodies and NZB BI specific T cells demonstrated reduced proliferation and cytokine production following BI priming as compared with non-transgenic controls (287,288). Thus, T cell clonal anergy induction appears to be intact in these mice.

Activation-induced cell death is another major mechanism by which autoreactive cells that become aberrantly activated in the immune system can be deleted. In both MRL.Fas\textsuperscript{lpr} and MRL.FasL\textsuperscript{gld} mice impairment of this process contributes to autoimmunity. These mouse strains develop an autoimmune disease characterized by massive lymphadenopathy, hypergammaglobulinemia and production of high titre pathogenic autoantibodies (reviewed in (289)). The lpr gene mutation encodes an aberrant form of Fas that results in disruption of Fas expression on the cell surface, whereas the gld gene mutation encodes a non-functional FasL due to a point mutation proximal to the extracellular C-terminus. Since Fas/FasL interaction plays a vital part in programmed cell death after T-cell activation, it is not surprising that the MRL.Fas\textsuperscript{lpr} and MRL.FasL\textsuperscript{gld} mice demonstrate impaired activation-induced cell death and massive lymphoproliferation as a result of accumulation of previously activated T cells. The presence of lpr T cells plays an important role in the production of autoantibodies by lpr B cells in lpr mice. In a mixed chimeric experiment, lethally irradiated B6.Fas\textsuperscript{lpr} mice were reconstituted with a mixture of bone marrow cells derived from B6 (Thy1.1) and
B6.Fas<sup>lpr</sup> (Thy1.2) mice followed by selective in-vivo depletion of lpr T cells using anti-Thy1.2 antibodies. Chimeras treated with anti-Thy1.2 antibodies exhibited marked depletion of lpr T cells with a significant reduction in the production of both IgM and IgG2a autoantibodies, indicating the importance of lpr T cell help in the generation of pathogenic autoantibodies (290).

**1.7.2.2 Deficiency and/or Impaired function of Regulatory T cells**

A 1996 study by Sakaguchi’s group showed that BALB/c mice neonatally thymectomized at age 3 days developed organ specific autoimmunity (291). It was suggested that neonatal thymectomy resulted in elimination of CD4<sup>+</sup>CD25<sup>+</sup> suppressor/regulatory T cells (Tregs), leading to aberrant activation of pre-existing self reactive T cells in the mice. It was later discovered that naturally occurring CD4<sup>+</sup> regulatory T cells express the forkhead/winged helix transcription factor, Foxp3, that programs the development and function CD4<sup>+</sup>CD25<sup>+</sup> Tregs. Mutations of the Foxp3 gene were found to be responsible for an X-linked recessive inflammatory disease, resulting in mortality in hemizygous Scurfy mutant males (Foxp3sf/Y) 16 to 25 days after birth (292). These male mice demonstrated a lack of Foxp3<sup>+</sup> Tregs, hypergammaglobulinemia, severe anemia, abnormal T cell activation and hyperproliferation of CD4<sup>+</sup> lymphocytes in response to low amounts of TCR stimuli.

Since the original discovery of Tregs multiple investigations have established the importance of Tregs in regulation of autoimmune diseases, including SLE (reviewed in (293-295)). In NZB/W mice the number of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells is reduced, although these cells appear to demonstrate normal suppressive function in vitro (296).
Further depletion of CD4⁺CD25⁺ Tregs in NZB/W mice by intravenous injection of anti-CD25 monoclonal antibody significantly increases production of proinflammatory cytokines and anti-ssDNA and dsDNA antibodies, resulting in an accelerated onset of glomerulonephritis. On the other hand, adoptive transfer of ex-vivo expanded CD4⁺CD25⁺ Tregs into NZB/W mice suppressed the onset of severe renal disease and prolonged the survival. Production of anti-dsDNA antibody, at least in part, appears to be regulated by these conventional CD4⁺CD25⁺Foxp3⁺ Tregs (297,298). Intravenous injection of NZB/W mice with high doses of an artificial peptide pConsensus (pCons) whose sequence is based on the shared complementary determining region (CDR1) of several murine anti-dsDNA antibodies prolongs survival, diminishes production of autoantibodies, and delays onset of renal diseases. By purifying CD4⁺CD25⁺ Tregs from pCons tolerized mice and coincubating them with B cells from untreated NZB/W mice, it was shown that peptide administration induced the expansion of pCons-reactive CD4⁺CD25⁺ regulatory T cells that inhibit the in vitro production of the anti-dsDNA by antibody forming cells (299).

In addition to naturally occurring CD4⁺CD25⁺Foxp3⁺ Tregs, TGF-β producing T helper type 3 cells (Th3) cells and T regulatory type 1 cells (Tr1) that preferentially produce IL-10 have been implicated in regulating the development of autoimmunity (300). These cells do not express Foxp3 and are generally considered as secondary suppressor T cells as they develop from peripheral CD4⁺CD25⁻ T cells. A recent study demonstrated that the severity of lupus inversely correlated with the IL-10 produced by Tr1 cells in a transgenic BXSB model (301). In another study, intravenous injection of high dose SmD1(83-119) peptide, a major T cell epitope, into NZB/W mice induced the
generation of Tr1 cells (CD4^+IL10^+) in the spleen and draining lymph nodes. These Tr1 cells inhibited anti-DNA autoantibody production through an IL-10 dependent pathway and prolonged the survival of NZB/W mice (302). However, another study has shown that when NZB/W mice were immunized with SmD1(83-119) peptide, elevated levels of pathogenic IgG anti-dsDNA and exacerbation of lupus were observed (278).

Mice deficient in TGF-β exhibit autoimmune phenotypes such as production of autoantibodies and glomerulonephritis similar to that observed in SLE patients, suggesting the importance of the cytokine TGF-β in the regulation of lupus pathogenesis. CD8^+ T cells isolated from NZB/W mice are deficient in production of TGF-β and exhibit altered proliferation upon stimulation (303). Administration of a pCons also induced expansion of TGF-β secreting CD8^+ T cells in NZB/W mice, and these cells were shown to directly suppress the proliferation of CD4^+ T cells and the production of anti-dsDNA antibodies by B cells (304).

1.7.2.3 Natural Killer T cells

Another category of regulatory T cells is natural killer T (NKT) cells. NKT cells are a unique T cell lineage that recognize glycolipid antigens in the context of CD1d, a non-classical MHC class I molecule. NKT cells have been shown to inhibit the progression of disease in a variety of autoimmune conditions (305-307). The proportion of NKT cells is reduced in a number of human autoimmune disorders including SLE, scleroderma, Sjogren’s syndrome, rheumatoid arthritis, multiple sclerosis and Type I diabetes mellitus (308,309). Similarly, in several animal models of autoimmune disease,
including the non-obese diabetic model of Type I DM, experimental autoimmune encephalomyelitis, and collagen-induced arthritis, deficiencies of NKT cells exacerbate disease while expansion and/or activation of NKT cells ameliorate disease. However, studies of the role of NKT cells in NZB, NZB/W and MRL mice have given rise to conflicting results. Young NZB and NZB/W mice have normal percentages of NKT cells. However, this population becomes expanded and activated with age and disease progression. Injection of α-galactosylceramide (α-GalCer), a glycolipid ligand that stimulates NKT cells and triggers a Th1 immune response in adult NZB/W mice, results in exacerbation of systemic autoimmunity (310). Treating NZB/W mice with anti-CD1 monoclonal antibody ameliorated the lupus phenotype and adoptive transfer of NKT cells from adult NZB/W mice to young pre-disease recipients induced renal disease (310,311). However, conflicting results regarding the roles of NKT cells in the regulation of autoimmunity were observed in MRL.Fas<sup>lpr</sup> mice. On one hand, the lupus prone MRL.Fas<sup>lpr</sup> mice have demonstrated a significant reduction in the proportion of NKT cells prior to the onset of autoimmunity (312). Strikingly, knocking out CD1d or β2-microglobulin in the MRL.Fas<sup>lpr</sup> background resulted in the exacerbation of inflammatory dermatitis but no change or even slightly less severe lupus nephritis (313,314). In addition, treatment of MRL.Fas<sup>lpr</sup> mice with α-GalCer fails to prevent any lupus like phenotypes, including renal nephritis, production of autoantibodies and splenomegaly despite significant increases in the proportion of NKT cells in treated mice (315). Similar conflicting results were observed in the BALB/c and SJL pristane-induced lupus mouse models. When these mice were injected with α-GalCer, expansion of predominantly IL-4 (Th2) or IFN-γ (Th1) producing NKT cells was observed in BALB/c
and SJL mice, respectively (316). This was associated with amelioration of disease in BALB/c mice but not SJL mice, suggesting that Th2 biased, IL-4 secreting NKT cells can prevent disease development whereas Th1 biased, IFN-γ producing NKT cells have the capability of exacerbating disease. These findings indicate the complexity when examining the roles of NKT cells in lupus pathogenesis, as both the number and Th1/Th2 balance of the NKT cells determine the regulatory ability of these cells.

Taken together the data suggest the presence of multiple regulatory T cell subsets abnormalities in lupus that could contribute to disease genesis. Understanding the mechanisms leading to these abnormalities would undoubtedly provide more in depth insights into the development of SLE and facilitate the discovery of novel therapeutic targets. In summary, T cells play an indispensible part in the pathogenesis of lupus. Our results and others have shown that functionally altered T cells activate functionally altered B cells, and vice versa, providing a positive feedback loop that lead to the amplification of the autoimmune phenotype (317). (Figure 1.2)
FIGURE 1.2 T-B cell interaction provides a positive feedback loop for the amplification of lupus pathogenesis. (Adapted from Shlomchik et al., Nature Immunology Review 2001 (317))
1.8 ROLE OF DENDRITIC CELLS IN THE PATHOGENESIS OF MURINE SLE

Dendritic cells (DCs) play an important role as the initiators and regulators of adaptive immune responses. As an initiator of adaptive immunity, immature DCs capture and process peptides derived from infectious pathogens and infected cells. With the presence of an inflammatory stimulus and proinflammatory cytokines such as IL-1, IL-6 and prostaglandins, these DCs become activated and upregulate costimulatory molecules on their cell surface (318). Activated DCs then present the processed antigens to naïve T cells thereby initiating immune responses. As a regulator of central tolerance, DCs are capable of presenting self antigens in the context of MHC molecules in the thymic medulla and the cortico-medullary junction to thymocytes, inducing clonal deletion of autoreactive thymocytes (reviewed in (319)). DCs can also act as inducers of T cell tolerance in the periphery. In the absence of acute infection or an inflammatory stimulus, DCs can capture and present self antigens in the absence of costimulation, thus inducing T cell anergy or deletion in the periphery (reviewed in (320)). Given the dual roles of DCs in regulation of immune responses, it is reasonable to speculate that abnormal DC activation might result in the loss of tolerance to self antigens in SLE.

1.8.1 Plasmacytoid Dendritic Cells in SLE

DCs can be classically divided into two distinct subsets: the conventional myeloid dendritic cells (cDCs or mDCs) and plasmacytoid dendritic cells (pDCs). B220+CD11cLow/NK1.1+/Ly6c+/CCR9+/PDCA-1+/Siglec-H+ pDCs are a rare cell type
derived from the common lymphoid progenitor, making up less than 0.5% of the lymphoid population in the peripheral blood (321-323). Developing in the bone marrow, pDCs migrate to and colonize T cell rich areas of secondary lymphoid organs around high endothelial venules (HEVs). These cells express TLR7 and TLR9 in the endosomal compartment. Various studies have demonstrated that TLRs initiate innate immune response by recognition of conserved structural motifs of microbial pathogens. Single stranded RNA has been identified as the natural agonist of TLR7 whereas TLR9 can recognize unmethylated microbial CpG DNA sequences (324,325). In viral infections signaling through these TLRs can trigger the production of type I interferons that play an important role in helping to clear the infection. In lupus patients, elevated levels of IFN-inducible genes are seen in the peripheral blood, which are thought to result from increased levels of Type I interferons. The levels of IFN-inducible gene expression in lupus correlate with serological and clinical manifestations of human SLE (326), particularly the presence of anti-dsDNA and -RNA binding protein antibodies, such as Ro, Sm, and RNP. Complexes between anti-DNA and –RNA binding protein antibodies and apoptotic debris have been shown that stimulate purified pDCs to secrete Type I interferons in a TLR- and FcγR-dependent fashion in-vitro. In normal individuals, peripheral blood mononuclear cells inhibit this complex induced secretion and this inhibition is deficient in lupus patients. At least in part this inhibition is mediated by secretion of TNF-α and IL-10, both of which have been shown to inhibit IFN-α secretion by pDC in-vitro. Thus, there appears to be a cross regulation between IFN-α and TNF-α, another proinflammatory cytokines that has been implicated in other autoimmune diseases such as rheumatoid arthritis (RA) and Crohn’s disease. Notably, a fraction of
RA patients that have been treated with TNF antagonists exhibit clinical symptoms of lupus that are reversible once the therapy is withdrawn (327,328) and viral hepatitis patients developed lupus like symptoms after undergoing IFN-α treatment (329).

In mice, pDCs and type I interferons have been shown to exacerbate lupus pathogenesis in various murine models of spontaneous lupus. Prolonged administration of IFN-α in young, pre-autoimmune NZB/W mice using an adenoviral vector leads to the rapid development of anti-dsDNA antibodies and lethal glomerulonephritis (330). NZB mice have an increased proportion of bone marrow pDCs that express significantly elevated level of TLR9 (331). When stimulated with type A CpG ODN 2216, a TLR9 ligand, these pDCs secreted a higher level of IFN-α in vitro. Notably, NZB mice lacking the type I interferon receptor (IFNAR) demonstrated reduced mortality, hemolytic anemia, and glomerulonephritis as well as diminished production of autoantibodies (332), further implicating IFN-α in disease severity. Similar findings were obtained in B6.Fas<sup>lpr</sup> mice. When injected with poly I:C, a potent inducer of Type I interferons, these mice developed severe renal disease, abnormal accumulation of activate lymphocytes and produced high titres of ANAs (333). IFN-α has pleomorphic effects on the immune system that serve to exacerbate SLE. Elevated level of IFN-α has been shown to induce the expression and release of autoantigens such as Ro52 in lupus patients (334-336). In addition, it can promote the differentiation of B cells into IgG antibody producing plasma cells (337). These IFN-α has been demonstrated to induce maturation and differentiation of monocytes into myeloid DCs that can act as potent APCs and lead to the activation of self reactive B and T cells (338,339). Furthermore, IFN-α can cause dysfunction of the regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cell population from patients with SLE (340). Taken together
these observations suggest that pDCs and production of type I interferons play pivotal roles in lupus pathogenesis.

1.8.2 Myeloid Dendritic Cells in SLE

In contrast to pDCs, the conventional myeloid CD11c+ subset (mDCs) are derived from a common myeloid progenitor. This lineage is comprised of peripheral circulating monocyte precursors, tissue-resident interstitial DCs and mucosal Langerhans cells. Myeloid DCs preferentially express TLR1, TLR2, TLR4, TLR8 on their cell surface and TLR3 intracellularly (341,342). Upon TLRs engagement, these cells become potent APCs through upregulation of costimulatory molecules such as CD80 and secrete a high levels of proinflammatory IL-12 that polarizes T cells towards a Th1 phenotype (343). When stimulated with LPS or apoptotic debris, mDCs produce a large amount of BAFF, an important cytokine in the pathogenesis of lupus that promotes BCR and CD40 dependent B cell proliferation (344,345).

Various studies have shown that the high levels of IFN-α in SLE patients’ sera is capable of inducing the differentiation and activation of monocytes into mDCs that can capture apoptotic debris and nucleosomes (346). These IFN-α induced mDCs can enhance the proliferation and activation of self reactive CD4+ and CD8+ T cells, thus triggering the development of autoimmunity. However, it is interesting to note that the induction of mDC differentiation by type I interferon requires the presence of intrinsic cellular defects as sera from SLE patients fail to induce the differentiation of DCs derived from the normal population (347). Myeloid DCs isolated from SLE patients exhibited
accelerated differentiation from monocytes, aberrant upregulation of costimulatory molecules including CD80, CD86 and MHC class II molecules, and increased production of the proinflammatory cytokine IL-8 (347). These mDCs have also been shown to enhance allogeneic T cell proliferation responses *in vitro*, thus further demonstrating the roles of these abnormal mDCs in the pathogenesis of lupus. Similar observations were made in B6.Sle1.Sle2.Sle3 triple congenic mice where increased number of CD11c⁺CD11b⁺ mDCs was observed in the bone marrow, spleen and lymph nodes when compared with B6 controls (348).

### 1.9 RATIONALE AND RESEARCH QUESTIONS

Previous experiments in our laboratory led to the identification of a major genetic region on chromosome 1 of the NZB mouse strain that was linked to production of autoantibodies and abnormal B cell activation (126). Congenic mice produced by backcrossing this interval (called B6.NZBc1(35-106) because the NZB interval extends from approximately 35-106 cM) onto the C57BL/6 background, made high titres of IgG anti-ssDNA and –chromatin autoantibodies, and developed renal disease (165). Characterization of the cellular phenotype of these mice revealed splenomegaly, an increased numbers of splenocytes, and an increased number and proportion of recently activated and memory B and T cells. Congenic mice carrying a shorter interval (82 to 106 cM) had also been generated in our laboratory. When compared with B6.NZBc1(35-106) mice, B6.NZBc1(82–106) mice had significantly less B and T cell activation and
significantly lower titres of anti-nuclear autoantibodies, suggesting the presence of at least two lupus susceptibility genes on NZB chromosome 1.

Contrasting the phenotypes of B6.NZBc1(35-106) and B6.NZBc1(82-106) congenic mice have lead us to develop the following hypotheses:

The 35-106 cM interval of NZB chromosome 1 contains at least two genes leading to bone marrow derived B and T cell defects resulting in the development of the autoimmune phenotype in NZB chromosome 1 congenic mice.

In order to characterize the intrinsic B and T cell defects within the 35-106cM interval on NZB chromosome 1 and examine their contributions to the development of lupus, I have proposed the following specific aims in the previous reclassification proposal:

(1) To confirm the presence of intrinsic T cell and B cell defect(s) within the 35-106 cM interval of NZB chromosome 1.

(2) To more precisely determine the location of lupus susceptibility genes within the 35-106 cM interval and examine the roles of possible candidate genes within these regions.

(3) To assess the function of T cells in B6.NZBc1(35-106) and B6.NZBc1(82-106) mice.
Experiments addressing these aims are outlined in Chapters 2 and 3 of the thesis.

In addition to the lupus susceptibility loci on New Zealand Black Mouse chromosome 1, our laboratory demonstrated linkage of a region on NZB chromosome 13 to elevated costimulatory molecule expression on B cells. To further explore this region a congeneric strain, called B6.NZBc13(24-73) mice, with an introgressed homozygous NZB chromosome 13 interval extending from 24 to 73 cM on C57BL/6 background was produced. These mice recapitulated most of the B cell phenotypic abnormalities in NZB mice and in addition demonstrated increased T cell activation, mild glomerulonephritis, high-titre IgM and IgG anti-chromatin antibody production, and a slightly expanded myeloid dendritic cell population (212). Further investigation through bone marrow reconstitution and mixed chimeric studies have indicated that the genetic locus/loci on chromosome 13 represents an extrinsic defect leading to polyclonal B cell activation (C. Loh et al., unpublished observation).

Epistatic interactions have been shown to play an essential role in amplifying the individual effects of independent susceptibility loci. I therefore produced bicongenic mice with both NZB chromosome 1 and 13 intervals, and hypothesized that:

**The lupus susceptibility loci on the 35-106 cM NZB chromosome 1 interval interacts with genetic loci located on 24-73 cM on NZB chromosome 13 leading to exacerbation of autoimmunity.**
Although this hypothesis was proven incorrect, as described in Chapter 4 these mice developed several novel phenotypes that have highlighted the complex regulation of IFNα production and disease severity.
CHAPTER 2

Functional Interplay Between Intrinsic B & T cell defects leads to amplification of autoimmune disease in NZB chromosome 1 congenic mice

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2.1- Abstract

Genetic loci on New Zealand Black (NZB) chromosome 1 play an important role in the development of lupus-like autoimmune disease. We have previously shown that C57BL/6 mice with an introgressed NZB chromosome 1 interval extending from ~ 35 to 106 cM have significantly more severe autoimmunity than mice with a shorter interval extending from ~ 82 to 106 cM. Comparison of the cellular phenotype in these mice revealed that both mouse strains had evidence of increased T cell activation, however activation was more pronounced in mice with the longer interval. Mice with the longer interval also had increased B cell activation, leading us to hypothesize that there were at least two independent lupus susceptibility loci on chromosome 1. In this study, we have used mixed hematopoietic radiation chimeras to demonstrate that autoimmunity in these mice arises from intrinsic B and T cell functional defects. We further show that a T cell defect, localized to the shorter interval, leads to spontaneous activation of T cells specific for nucleosome histone components. Despite activation of self-reactive T cells in mixed chimeric mice, only chromosome 1 congenic B cells produce anti-nuclear antibodies and undergo class switching, indicating impaired B cell tolerance mechanisms. In mice with the longer chromosome 1 interval an additional susceptibility locus exacerbates autoimmune disease by producing a positive feedback loop between T and B cell activation. Thus T and B cell defects act in concert to produce and amplify the autoimmune phenotype.
2.2- Introduction

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease of unknown etiology that mainly affects women of childbearing age. One of the hallmarks of this disease is the loss of tolerance to self-antigens, particularly nuclear antigens (1). This loss of tolerance leads to production of autoantibodies (ANAs) directed against nuclear antigens, such as chromatin, and results in the formation of immune complexes. Deposition of immune complexes in the glomeruli, skin, and other organs induces tissue damage resulting in the manifestations of disease, which include skin rash, arthritis, and glomerulonephritis (349). Although the genes responsible for development of SLE in humans have not been clearly identified, there is convincing evidence that SLE has a strong, but complex, genetic basis (350).

Identification of the immune abnormalities that lead to the development of SLE has been greatly aided by the study of lupus-prone mouse strains. New Zealand Black (NZB) mice develop a lupus-like autoimmune disease characterized by production of anti-ssDNA and anti-red blood cell antibodies leading to hemolytic anemia (121). Mapping studies have revealed that susceptibility to lupus in NZB mice is polygenic with multiple genes contributing to the generation of high affinity autoantibodies, nephritis, and mortality in various crosses (124-126,153,156,351-354). In particular, genetic loci on NZB chromosome 1 have been shown in multiple crosses to play a prominent role in disease susceptibility (125,126,153,156,351,352). Indeed, in one cross between NZB and B6.H2z mice, a region on distal NZB chromosome 1 was found, in combination with H2z, to confer >90% of the susceptibility to nephritis and autoantibody production (153,352).
The presence of a lupus susceptibility gene(s) on distal NZB chromosome 1 was confirmed by Rozzo et al, who showed that B6 congenic mice with an NZB interval extending from ~79 to 109 cM, produced IgG anti-nuclear antibodies (164). Three candidate genes have been proposed to contribute to the development of autoimmunity in this interval: *Iffi202* (164), *Fcgr2b* (181,182) and the SLAM/CD2 cluster (170). However, work by ourselves and others suggested that it was unlikely that this interval contained all of the lupus susceptibility genes located on NZB chromosome 1 (126,351). For example, Kono et al. showed linkage between a NZB region around 67 cM and anti-chromatin antibody production and splenomegaly in a (NZB x New Zealand White (NZW))F2 intercross (156). In our mapping study of (B6 x NZB)F2 intercross mice, IgG anti-ssDNA antibody production was linked to a broad region on NZB chromosome 1 with 2 shallow LOD score peaks at 63 cM and 92 cM, and a similar area of broad linkage was demonstrated for several B cell activation phenotypes, including increased expression of B7.1, B7.2, ICAM-1, and CD44 (126). Indeed, the peak LOD scores for IgG anti-ssDNA antibody production, ICAM-1, and CD44 were all located in the 63 to 76 cM region.

To investigate the presence of additional susceptibility genes on NZB chromosome 1, we generated congenic mice with NZB chromosome 1 intervals of various lengths and compared their cellular and autoimmune phenotype (165). We found that mice with a congenic interval extending from ~35 to 106 cM had a significantly more severe autoimmune phenotype than mice with an interval extending from ~82 to 106 cM, confirming the presence of at least two independent lupus susceptibility loci on NZB chromosome 1. Mice with the shorter interval (labeled B6.NZBc1S in this report)
had evidence of increased T cell activation with an increased proportion of memory T cells, whereas mice with the longer interval (B6.NZBc1L) had more robust T cell activation and an increased proportion of activated B cells. The increased T cell activation in B6.NZBc1L mice, as compared to B6.NZBc1S mice, was B cell dependent, leading us to hypothesize that the genetic locus located within the shorter interval predominantly affected T cell function, while that located in the longer interval altered B cell function.

In this study we have further investigated the nature and location of the immune abnormalities in these mice. By generating mixed hematopoietic radiation chimeras, we show that autoimmunity in B6.NZBc1L mice results from intrinsic B and T cell defects. We further show that the susceptibility locus located within the shorter interval leads to spontaneous activation of T cells with specificity for nucleosome histone components. Despite the presence of activated self-reactive T cells in mixed chimeric mice, tolerance in B6-derived B cells is sufficient to prevent autoantibody production because only congenic B cells produce anti-nuclear antibodies. Thus, in mice with the shorter interval T and B cell defects must act together to produce the autoimmune phenotype. In mice with the longer NZB interval, autoimmunity is exacerbated by the presence of an additional susceptibility locus that leads to enhanced T and B cell activation through generation of a positive feed back loop. The data provide new insights into the nature of the immune defects that produce autoimmunity in NZB chromosome 1 congenic mice.
2.3- Methods

2.3.1- Mice

C57BL/6 (B6), C57BL/6.Thy1<sup>a</sup>IgH<sup>a</sup>Gpi<sup>a</sup> (denoted here as B6.Thy1<sup>a</sup>IgH<sup>a</sup>), and B6.129S2-Tcra<sup>tm1Mom</sup> (B6.TCRα-/-) breeders were purchased from the Jackson Laboratory (Bar Harbor, ME). NZB mice were purchased from Harlan-Sprague-Dawley (Indianapolis, IN). Congenic mice were produced by backcrossing mice with NZB chromosome 1 intervals onto the B6 background, using the speed congenic technique. Mice were typed at each successive generation using polymorphic microsatellite markers that discriminate between NZB and B6 DNA. Markers were spaced at ~ 20 cM intervals throughout the genome except for regions containing lupus susceptibility genes where more densely spaced markers were used. Fully backcrossed mice were obtained in 6 to 7 generations and intercrossed to produce congenic mice that were homozygous for NZB chromosome 1 intervals. Mice used in this study, denoted B6.NZBc1L and B6.NZBc1S for simplicity, correspond to the B6.NZBc1(35-106) and B6.NZBc1(85-106) mice published previously (165). B6.NZBc1L and B6.NZBc1S mice have introgressed NZB intervals extending from between D1Mit21 (32.8 cM) and D1Mit303 (34.8 cM) or D1Mit396 (79 cM) and D1Mit159 (81.6 cM) to between D1Mit223 (106cM) and D1Mit210 (109 cM). The borders of these intervals have been refined from our previous publication by identification and testing of additional polymorphic primers. B6.NZBc1L and B6.NZBc1S mice expressing transgenes encoding IgM/IgD H and L chains specific for hen egg white lysozyme (anti-HEL Ig transgenic (Tg), MD4, (355)) were produced by backcrossing the anti-HEL Ig transgene from B6 anti-HEL Ig Tg mice (purchased from the Jackson Laboratory). Offspring were genotyped by PCR, using primers specific for
the V region of the Ig H chain. B6.NZBc1L.TCRα-/ mice were produced by backcrossing the gene deletion onto the B6.NZBc1L background and then intercrossing to produce knockout mice. TCRα knockout mice were genotyped by PCR, using neo and TCRα specific primers. The mice were housed in microisolators in the animal facility at the Toronto Western Hospital and were specific-pathogen free. All of the mice used in this study were female.

2.3.2- Generation of bone marrow reconstituted and mixed chimeric mice

For bone marrow reconstitution experiments, 6 to 8-week-old female B6.Thy1aIgHα recipient mice were put on sterilized acid water at least 1 week before reconstitution. On the day of cell transfer, recipient mice were given two doses of gamma radiation (525 rad) separated by 2h and injected, through the tail vein, with 1 x 10^7 T cell-depleted bone marrow cells from B6 or B6.NZBc1L mice. For mixed chimeras, 6 to 8-week-old female B6 mice were treated, as above, and injected with a mixture of T cell-depleted bone marrow from B6.Thy1aIgHα mice and B6 or B6.NZBc1L mice at a ratio of 3:1 or 1:1. Recipients were sacrificed and analyzed 4 or 7 months later.

2.3.3- Flow cytometry analysis

RBC-depleted splenocytes (5 x 10^5) were incubated with 10 μg/ml mouse IgG (Sigma-Aldrich, St Louis, MO) for 15 min to block Fc receptors and stained with various combinations of directly-conjugated mAbs. Following washing, allophycocyanin-conjugated streptavidin (BD PharMingen, San Diego, CA) was used to reveal biotin-conjugated Ab staining. Dead cells were excluded by staining with 0.6 μg/ml propidium
iodide (Sigma-Aldrich). Flow cytometry of the stained cells was performed using a dual laser FACScalibur (BD Biosciences, Mountain View, CA) and analyzed using Cell Quest (BD Biosciences) software. Live cells were gated on the basis of PI uptake and scattering characteristics with 10,000 events being acquired for each sample. The following directly conjugated mAbs were purchased from BD Pharmingen: biotin anti-CD11c (N418), biotin anti-CD11b (Mac1), biotin anti-CD4 (L3T4), biotin anti-CD8 (53-6.7), biotin anti-CD62L (MEL-14), biotin anti-B220 (RA3-6B2), biotin anti-Thy1.1 (HIS51), biotin anti-Thy1.2 (53-2.1), biotin anti-IgM\(^a\) (DS-1), biotin anti-IgM\(^b\) (AF6-78), biotin anti-IgG2a\(^a\) (8.3), biotin anti-IgG2a\(^b\) (5.7), PE anti-B7.1 (16-10A1), PE anti-B7.2 (GL1), PE anti-CD3 (145-2C11), PE anti-CD69 (H1.2F3), PE anti-CD44 (IM7), PE anti-NK1.1 (PK136), PE anti-CD4 (H129.19), FITC anti-CD3 (145-2C11), FITC anti-CD4 (L3T4), FITC anti-CD8 (53-6.7), FITC anti-CD21/CD35 (7G6), FITC anti-CD25 (7D4), FITC anti-B220 (RA3-6B2), FITC anti-TCR\(\beta\) (H57-597) and FITC anti-TCR\(\delta\gamma\) (GL3). FITC anti-CD62L was purchased from Cedarlane (Hornby, Ontario, Canada). Biotinylated HEL was prepared using an EZ-Link Sulfo-NHS-LC Biotinylation kit (Pierce, Rockford, IL). All isotype controls, with the exception of hamster IgG controls (BD Pharmingen), were purchased from Cedarlane.

2.3.4- APC isolation

Single-cell suspensions of splenocytes were isolated from 2 to 3 month old B6 mice by pressing through a nylon mesh. RBCs were removed by lysis in Geys’ solution. The cell suspension was then incubated with purified anti-Thy1.2 (HO-13-4) and anti-CD4 (GK1.5) mAb followed by guinea pig complement (Cedarlane) to remove T cells.
Following lysis, the resultant cell population (<1% CD4⁺ T cell contamination) was resuspended in RPMI1640 containing 0.5% normal mouse serum (NMS).

2.3.5- **CD4⁺ T cell isolation**

Single-cell suspensions of RBC-depleted splenocytes were suspended in 2% FBS/PBS and incubated with biotinylated anti-B220, -I-A^d^, -CD8, -Mac1, -CD11c and –CD24 antibodies on ice for 30 minutes. Excess antibodies were removed by washing and the cells were then resuspended in 2% FBS/PBS at a concentration of 100 x 10⁶ cells per ml. Streptavidin-conjugated Dynabeads (M-280, DYNAL Biotech, Oslo, Norway) were washed with 2% FBS/PBS to remove azide and resuspended at 160 x 10⁶ beads/ml in PBS. An equal volume of washed Dynabeads was added to the cell suspension and incubated at room temperature for one hour. CD4⁺ T cells were then purified by negative selection with a magnet and resuspended in 0.5% NMS/RPMI1640. The resultant cell population contained >80% CD4⁺ T cells.

2.3.6- **T cell proliferation and cytokine assays**

T cell-depleted splenic APCs, isolated from B6 mice, were irradiated with 2000 rads and then incubated for 1 h at 37°C with media alone or containing 1μg/ml total bovine histones, H1, H2A, H2B, H3, or H4 (Roche, Quebec, Canada). Excess antigen was removed by washing with PBS and the cells resuspended in 0.5% NMS/RPMI1640. Antigen-pulsed APCs were cultured at 0.5x10⁶ cells per well together with purified CD4⁺ T cells (0.5 x 10⁶ per well) for 48 h at 37°C in 96 well flat bottom plates. Anti-CD3 (5 μg/ml) or conA (5 μg/ml) were added to control wells at the time of plating. Proliferation
was measured by $[^3\text{H}]$-thymidine incorporation after a 16 h pulse with 1 $\mu$Ci/well. Uptake of $[^3\text{H}]$-thymidine was quantified by a scintillation counter and expressed as mean cpm ± SD of triplicate wells. For each antigen condition a stimulation index was calculated by dividing the mean cpm in the presence of antigen by the mean cpm in the absence of antigen.

IL-4 and IFN-γ levels in tissue culture supernatants were measured at 48 hr. Anti-IL4 and -IFN-γ capture antibodies, biotinylated-anti-IL4 and -IFN-γ detection antibodies, and recombinant IL-4 and IFN-γ were purchased from BD Pharmingen. Assays were performed as per the manufacturer’s recommendations. The concentration of cytokine in each supernatant was calculated from a standard curve with log-log plot of absorbance versus concentration of recombinant cytokine preparation.

2.3.7- Measurement of Ab production

Levels of IgM, IgM$^a$, IgM$^b$, IgG, IgG2a, IgG2a$^a$ and IgG2a$^b$ anti-chromatin and -ssDNA Abs in the sera were measured by ELISA. ssDNA was prepared by boiling dsDNA (isolated from calf thymus DNA) for 10 minutes and quick cooling on ice. H1-stripped chromatin was prepared from chicken RBC as described (356). ELISA plates were coated overnight with either ssDNA (20 μg/ml) or chromatin (8 μg/ml) in PBS, washed with 0.05% Tween 20/PBS, and blocked with 2% BSA/PBS for 1h at room temperature. After washing, serum samples were diluted 1/100 in 2% BSA/0.05% Tween 20/PBS, added to ELISA plates, and incubated for 1h at room temperature. The presence of bound Abs was detected by adding alkaline-phosphatase-conjugated anti-IgM, IgG, or -IgG2a (Caltag, Burlingame, CA) as secondary reagents. For allotype-specific ELISAs,
biotinylated anti-IgG2a\textsuperscript{a} and -IgG2a\textsuperscript{b}, or –IgM\textsuperscript{a} and IgM\textsuperscript{b} mAb (BD Pharmingen) were used and detected with alkaline-phosphatase-conjugated streptavidin (BD Pharmingen). To control for differences in the ability of the allotype-specific antibodies to detect bound antibody, control sera were run on each assay that were of ‘\textit{a}’ (MRLlpr/lpr) or ‘\textit{b}’ (B6.NZBc1L) allotype, and the results for allotype-specific assays were normalized to those seen with assays using anti-IgG2a or -IgM antibodies that detected both allotypes.

2.3.8- Immunofluorescence Staining of Tissue Sections.

Spleens were snap frozen in OCT compound (Sakura Finetek, Torrance, CA) at the time of sacrifice. Cryostat sections (5 \(\mu\)m) were fixed in acetone, washed with PBS, and blocked with 5\% normal goat serum/PBS. Sections were stained with biotinylated-PNA (Sigma-Aldrich) with or without FITC anti-CD21 to detect germinal centres. For allotype specific antibody staining of germinal centres, sections were stained with biotinylated anti- IgM\textsuperscript{a}, - IgM\textsuperscript{b}, - IgG2a\textsuperscript{a}, or – IgG2a\textsuperscript{b} mAb together with FITC anti-CD21. Biotin staining was revealed using rhodamine-conjugated streptavidin as a secondary reagent (Molecular Probes, Eugene, OR). Stained sections were mounted with Mowiol (Calbiochem, La Jolla, CA) and tissue fluorescence visualized using a Zeiss Axioplan 2 imaging microscope (Oberkochen, Germany). Digital images were obtained using the manufacturer’s imaging system. Germinal centres were scored as positive for IgM\textsuperscript{a}, IgM\textsuperscript{b}, IgG2a\textsuperscript{a}, and IgG2\textsuperscript{b} cells, when one or more cells could be clearly localized to the PNA\textsuperscript{+} region of the germinal centre using CD21 staining to align serial sections. Cells that stained with anti-allotype antibody but were CD21\textsuperscript{bright}, and thus likely to be follicular dendritic cells, were excluded from the analysis.
2.3.9- *Statistics*

Comparisons of differences between groups of mice were performed using a Mann-Whitney non-parametric test with the exception of IFN-γ levels where a Wilcoxon two-sample test was used. P-values < 0.05 were considered to be significant.
2.4- Results

2.4.1- Reconstitution of B6 mice with bone marrow cells from B6.NZBc1L mice transfers the autoimmune phenotype

To determine whether the genetic polymorphism(s) leading to autoimmunity in B6.NZBc1L mice affect bone marrow derived immune cell populations, bone marrow cells from B6.NZBc1L (Thyl\textsuperscript{b}IgH\textsuperscript{b}) or normal B6 (Thyl\textsuperscript{b}IgH\textsuperscript{b}) mice were injected into lethally irradiated B6.Thyl\textsuperscript{a}IgH\textsuperscript{a} mice and the recipients were analyzed 6 months post reconstitution. In these mice the majority of T and B cells were derived from the donor bone marrow (% IgM\textsuperscript{b+}B220\textsuperscript{+} cells > 90%; % Thy1\textsuperscript{b+}CD4\textsuperscript{+} cells >90%).

In B6.NZBc1L mice, autoimmunity is characterized by increased production of IgG anti-ssDNA and –chromatin antibodies, together with markers of increased T and B cell activation (165). As shown in Table 2.1, 6 month old B6.NZBc1L mice analyzed in tandem with reconstituted mice had the same phenotype that we have previously reported for 4 month old mice including: splenomegaly, increased proportions of activated (CD69\textsuperscript{+}) and costimulatory molecule expressing B cells, and increased proportions of activated (CD69\textsuperscript{+}) and memory (CD44\textsuperscript{hi}CD62L\textsuperscript{lo}) CD4\textsuperscript{+}cells (165). All of these phenotypes were recapitulated in mice reconstituted with B6.NZBc1L bone marrow. Transfer of B6.NZBc1L bone marrow also resulted in the same altered distribution of peripheral B cells subsets as seen in B6.NZBc1L mice, with increased proportions of CD21\textsuperscript{lo}CD23\textsuperscript{lo} and follicular B cells, and decreased proportions of T2 and marginal zone B cells (data not shown). As in our previous study, the increased costimulatory molecule expression was restricted to (for B7.1), or predominantly in (for B7.2 and ICAM-1), the CD21\textsuperscript{lo} B cell population (Table 2.1 and data not shown).
**TABLE 2.1**

Table I. *The autoimmune phenotype in B6.NZBc1L mice can be reproduced by reconstituting lethally irradiated B6.Thy1\(^a\) IgH\(^a\) mice with B6.NZBc1L bone marrow* \(^1\)

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>B6.NZBc1L</th>
<th>B6⇒B6.Thy1(^a)IgH(^a)</th>
<th>B6.NZBc1L⇒B6.Thy1(^a)IgH(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N = 5)</td>
<td>(N = 5)</td>
<td>(N = 3)</td>
<td>(N = 7)</td>
</tr>
<tr>
<td>spleen weight (mg)</td>
<td>70.8 ± 9.39</td>
<td>125.6 ± 2.70 *</td>
<td>50.33 ± 8.02</td>
<td>93.86 ± 21.33 *</td>
</tr>
<tr>
<td># splenocytes (x 10(^6))</td>
<td>44.2 ± 8.11</td>
<td>91.40 ± 19.05 **</td>
<td>38.0 ± 2.65</td>
<td>52.29 ± 8.28 *</td>
</tr>
<tr>
<td>% B220(^+)</td>
<td>57.29 ± 5.33</td>
<td>54.31 ± 5.64</td>
<td>49.18 ± 5.05</td>
<td>61.83 ± 3.53 *</td>
</tr>
<tr>
<td>% CD4(^+)</td>
<td>18.37 ± 2.54</td>
<td>19.88 ± 2.67</td>
<td>17.07 ± 4.74</td>
<td>18.64 ± 2.24</td>
</tr>
<tr>
<td>% CD8(^+)</td>
<td>12.3 ± 2.02</td>
<td>9.28 ± 0.23 *</td>
<td>11.26 ± 4.88</td>
<td>9.36 ± 2.37</td>
</tr>
<tr>
<td>% B220(^+) CD69(^+)</td>
<td>6.12 ± 0.84</td>
<td>14.40 ± 4.46 **</td>
<td>6.53 ± 1.93</td>
<td>19.04 ± 3.62 *</td>
</tr>
<tr>
<td>% B220(^+) B7.1(^+)</td>
<td>9.24 ± 0.65</td>
<td>12.06 ± 5.53</td>
<td>7.24 ± 2.09</td>
<td>11.49 ± 2.10 *</td>
</tr>
<tr>
<td>B220(^+) MFI B7.2</td>
<td>50.24 ± 17.54</td>
<td>74.16 ± 16.69</td>
<td>40.89 ± 2.40</td>
<td>65.33 ± 16.66 *</td>
</tr>
<tr>
<td>B220(^+) MFI ICAM-1</td>
<td>350.3 ± 106.9</td>
<td>544.2 ± 99.14 *</td>
<td>310.6 ± 26.03</td>
<td>503.4 ± 111.1 *</td>
</tr>
<tr>
<td>% B7.1(^+) CD21(^{lo}) B220(^+)</td>
<td>14.36 ± 3.54</td>
<td>37.54 ± 6.36 **</td>
<td>24.91 ± 2.83</td>
<td>34.4 ± 8.41 *</td>
</tr>
<tr>
<td>MFI B7.2 CD21(^{lo}) B220(^+)</td>
<td>43.74 ± 8.15</td>
<td>65.63 ± 7.47 **</td>
<td>40.09 ± 3.69</td>
<td>56.35 ± 10.52 *</td>
</tr>
<tr>
<td>MFI ICAM-1 CD21(^{lo}) B220(^+)</td>
<td>355.9 ± 83.35</td>
<td>515.1 ± 71.28 *</td>
<td>355.8 ± 8.46</td>
<td>459.6 ± 84.52 *</td>
</tr>
<tr>
<td>% CD4(^+) CD69(^+)</td>
<td>24.21 ± 4.66</td>
<td>40.83 ± 9.24 **</td>
<td>24.89 ± 6.47</td>
<td>43.2 ± 10.64 *</td>
</tr>
<tr>
<td>% CD4(^+) CD44(^{hi})CD62L(^{lo})</td>
<td>30.55 ± 3.25</td>
<td>57.39 ± 12.68 *</td>
<td>30.46 ± 7.51</td>
<td>59.65 ± 9.7 *</td>
</tr>
</tbody>
</table>

\(^1\) Results are mean ± SD. Numbers in brackets denote the number of female mice examined in each group. Wild type mice were examined at 6 months of age whereas reconstituted mice were examined 6 months post reconstitution. Significance level for comparison of B6.NZBc1L with B6 controls or B6.NZBc1L⇒B6.Thy1\(^a\)IgH\(^a\) with B6⇒B6.Thy1\(^a\)IgH\(^a\) mice, as determined by Mann-Whitney non-parametric test.

* p < 0.05, ** p < 0.01
Mice reconstituted with B6.NZBc1L bone marrow also had significantly elevated levels of IgG anti-chromatin (OD ± SD; B6.NZBc1L = 0.371 ± 0.261, B6 = 0.006 ± 0.002, \( p = 0.017 \)) and –ssDNA antibodies (OD ± SD; B6.NZBc1L = 0.224 ± 0.159, B6 = 0.018 ± 0.019, \( p = 0.033 \)), as compared to mice reconstituted with B6 bone marrow. Thus, the immune defects leading to abnormal lymphocyte activation and autoantibody production in NZB chromosome 1 congenic mice affect bone marrow derived cell populations.

2.4.2- B6.NZBc1L mice have intrinsic B and T cell functional defects

To investigate the roles of intrinsic B and T cell functional abnormalities in production of the autoimmune phenotype in NZB chromosome 1 congenic mice, chimeric mice with a mixture of bone marrow from B6.NZBc1L and B6.Thyl\(^a\)Igh\(^a\) mice were produced. Mice receiving a mixture of bone marrow from normal B6 and B6.Thyl\(^a\)Igh\(^a\) were used as controls. Bone marrow mixtures were injected into 6 to 8 week old lethally irradiated B6 mice at a 3:1 or 1:1 ratio (Thyl\(^a\)Igh\(^a\):Thyl\(^b\)Igh\(^b\)), and the recipients assessed at 4 (3:1 ratio) or 7 (1:1 ratio) months post-reconstitution. Allotype-specific mAb for Thy1 and IgM were used to discriminate between B6 and B6.NZBc1L T and B cells.

Consistent with previous experiments using bone marrow from New Zealand Mixed (NZM)-derived congenic mouse strains (201,234), chimeric mice injected with a 3:1 ratio of Thy1\(^a\)Igh\(^a\):Thy1\(^b\)Igh\(^b\) bone marrows had similar proportions of Thy1\(^a\) and Thy1\(^b\) cells (B6 + B6.Thyl\(^a\)Igh\(^a\), %Thy1\(^a\) = 10.09 ± 0.84, %Thy1\(^b\) = 8.106 ± 1.67; B6.NZBc1L + B6.Thyl\(^a\)Igh\(^a\), %Thy1\(^a\) = 14.14 ± 1.289, %Thy1\(^b\) = 11.68 ± 2.997), and
IgM\textsuperscript{a} and IgM\textsuperscript{b} (B6 + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a}, \%IgM\textsuperscript{a} 42.74 ± 4.221, \%IgM\textsuperscript{b} 34.73 ± 4.348; B6.NZBc1L + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a}, \%IgM\textsuperscript{a} 36.98 ± 3.787, \%IgM\textsuperscript{b} 35.85 ± 4.857). Further analysis of these mice revealed that B6.NZBc1L-derived B and T cells retained their abnormal activation phenotype (Figure 2.1). In mice with a mixture of B6.NZBc1L and B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} bone marrow, the proportion of Thy1\textsuperscript{b} T cells that were CD69\textsuperscript{+} and CD44\textsuperscript{hi}CD62L\textsuperscript{lo} was significantly increased when compared to Thy1\textsuperscript{a} T cells and compared to Thy1\textsuperscript{b} T cells in mice with a mixture of B6 and B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} bone marrow (Figure 2.1A and B), indicating that the abnormal T cell activation in B6.NZBc1L mice results from an intrinsic T cell defect.

In contrast to the findings observed for T cells, the proportions of CD69\textsuperscript{+} IgM\textsuperscript{a} and IgM\textsuperscript{b} B cells in chimeric mice with a mixture of B6.NZBc1L and B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} bone marrow were comparable and significantly elevated when contrasted with their counterparts in mice with a mixture of B6 and B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} bone marrow (Figure 2.1C). Nevertheless, costimulatory molecule expression was higher in IgM\textsuperscript{b} than IgM\textsuperscript{a} B cells. In the total B cell population this achieved statistical significance for B7.2 and ICAM-1 (%B7.1\textsuperscript{+}, IgM\textsuperscript{a} = 16.03 ± 3.23, IgM\textsuperscript{b} = 19.81 ± 4.26, p = NS; MFI B7.2, IgM\textsuperscript{a} = 11.90 ± 1.31, IgM\textsuperscript{b} = 15.38 ± 1.23, p = 0.0002; MFI ICAM-1, IgM\textsuperscript{a} = 206.4 ± 21.91, IgM\textsuperscript{b} = 279.2 ± 67.88, p = 0.015). Staining with anti-CD21 to discriminate between peripheral B cell subsets revealed that costimulatory molecule expression on IgM\textsuperscript{b} cells was significantly elevated as compared to IgM\textsuperscript{a} cells only in the CD21\textsuperscript{lo} population (Figure 2.1D-F). Although costimulatory molecule expression was increased somewhat in the CD21\textsuperscript{int} and CD21\textsuperscript{hi} populations in mice reconstituted with B6.NZBc1L + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} bone marrow as compared with mice reconstituted with B6 +
B6.Thyl\textsuperscript{a}IgH\textsuperscript{a} bone marrow, this did not differ between IgM\textsuperscript{a} and IgM\textsuperscript{b} B cells. Thus, B6.NZBc1L mice have an intrinsic B cell defect that leads to altered upregulation of costimulatory molecules in the CD21\textsuperscript{hi} population.
FIGURE 2.1 Splenic B and T cell activation in a and b allotype cells from mixed chimeric mice. Freshly isolated splenocytes from 4 month old chimeric mice, receiving a mixture of B6 + B6.Thy1aIgHa bone marrow ( ▲ = a allotype cells, △ = b allotype cells) or B6.NZBc1L + B6.Thy1aIgHa bone marrow ( ● = a allotype cells, or ○ = b allotype cells), were stained with anti-IgMa or –IgMb, anti-CD21, and anti-B7.1, –B7.2, –ICAM-1 or –CD69 antibodies to assess B cell activation. T cell activation was investigated by staining with anti-Thy1a or –Thy1b, and anti-CD44 and –CD62L, or anti-CD4 and -CD69, antibodies. Shown are the (A) percent CD69+ cells within the CD4+ subset, (B) percent CD62LloCD44hi cells (C) percent CD69+ cells, (D) percent B7.1+ cells within the CD21lo subset, (E) MFI for B7.2 staining in the CD21lo subset, or (F) MFI for ICAM-1 in the CD21lo subset, gating independently on Thy1a or Thy1b (A,B), or IgMa or IgMb (C-F), positive populations. Each symbol represents the determination from an individual mouse. Horizontal lines indicate the mean for each population examined.
We next assessed whether B6 and B6.NZBc1L B cells equivalently differentiate into antibody producing cells. In B6.NZBc1L mice, IgG autoantibodies of the IgG2a, but not IgG1, subclass are produced (data not shown). As shown in Figure 2.2A, chimeric mice receiving B6.NZBc1L + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} bone marrow cells, also had significantly increased levels of IgG2a anti-ssDNA (p < 0.05) and -chromatin (p < 0.005) antibodies as compared to B6 controls and B6 + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} bone marrow chimeric mice. Furthermore, the levels of autoantibodies produced were comparable to those seen in 16-week-old B6.NZBc1L mice. Characterization of the origin of these antibodies using allotype specific antibodies revealed that only ‘b’ allotype IgG2a anti-ssDNA (p < 0.05) and -chromatin (p < 0.005) antibodies were produced (Figure 2.2B). Consistent with our previous results, unmanipulated B6.NZBc1L and chimeric mice receiving B6.NZBc1L + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} bone marrow cells did not produce significantly increased levels of IgG2a anti-dsDNA antibodies (OD ± SD; IgG2a anti-dsDNA, B6 = 0.076 ± 0.053, B6.NZBc1L = 0.135 ± 0.132, p = NS; B6 + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} = 0.098 ± 0.027, B6.NZBc1L + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} = 0.237 ± 0.168, p = NS). Nevertheless, ‘b’ allotype IgG2a anti-dsDNA levels were increased slightly in mice reconstituted with B6.NZBc1L + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} bone marrow cells (OD ± SD; IgG2a anti-dsDNA; ‘a’ allotype, B6 + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} = 0.053 ± 0.020, B6.NZBc1L + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} = 0.034 ± 0.014, p = NS; ‘b’ allotype, B6 + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} = 0.032 ± 0.015, B6.NZBc1L + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} = 0.150 ± 0.170, p = 0.03). Similar results were obtained for mice analyzed at 7 months post-reconstitution (data not shown).

Although B6.NZBc1L mice produce relatively low titres of IgM anti-nuclear antibodies, significantly elevated levels of IgM\textsuperscript{b}, but not IgM\textsuperscript{a}, anti-ssDNA and –
chromatin antibodies could be detected in mice reconstituted with B6.NZBc1L + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} bone marrow cells (OD \pm SD; anti-ssDNA, IgM\textsuperscript{a} = 0.054 \pm 0.027, IgM\textsuperscript{b} = 0.562 \pm 0.361, p = 0.0002; anti-chromatin, IgM\textsuperscript{a} = 0.038 \pm 0.027, IgM\textsuperscript{b} = 0.486 \pm 0.49, p = 0.0003). Thus, despite the presence of T cells that can provide help for antibody production in B6.NZBc1L + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} chimeric mice, only B6.NZBc1L B cells can differentiate into autoantibody producing cells or undergo class-switching.
FIGURE 2.2 Autoantibody levels in mixed chimeric mice. Serum samples from (A) 4 month old B6 (▲), B6.NZBc1L (●), or chimeric mice receiving either a mixture of B6 + B6.Thy1^aIgH^a bone marrow (△) or B6.NZBc1L + B6.Thy1^aIgH^a bone marrow (○) were assayed for the presence of IgG2a anti-ssSNA or chromatin Abs by ELISA. (B) Allotype-specific ELISA assays were used to detect the presence of IgG2a^a and IgG2a^b anti-ssDNA and anti-chromatin antibodies in chimeric mice receiving a mixture of B6 + B6.Thy1^aIgH^a bone marrow (▲ = IgG2a^a and △ = IgG2a^b) or B6.NZBc1L + B6.Thy1^aIgH^a bone marrow (● = IgG2a^a and ○ = IgG2a^b). Each symbol represents the determination from an individual mouse. Horizontal lines indicate the mean for each population examined. Significantly higher titres of “b” allotype anti-ssDNA (p < 0.05) and anti-chromatin (p < 0.005) antibodies were detected in mice receiving a combination of B6.NZBc1L + B6.Thy1^aIgH^a bone marrow.
We have previously shown that B6.NZBc1L mice have a markedly increased number of germinal centres as compared to B6 and B6.NZBc1S mice (165). This phenotype was shared by B6.NZBc1L + B6.Thy1\(^a\)IgH\(^a\) chimeric mice (Figure 2.3A-C), who had a significantly increased number of germinal centres as compared to mice with B6 + B6.Thy1\(^a\)IgH\(^a\) bone marrow cells (B6 + B6.Thy1\(^a\)IgH\(^a\) = 5.80 ± 2.59, B6.NZBc1L + B6.Thy1\(^a\)IgH\(^a\) = 23.50 ± 11.59, p < 0.005). To determine whether B6 and B6.NZBc1L B cells are equivalently recruited into germinal centres, spleen sections were stained with anti-CD21 and PNA to reveal germinal centres, and then serial sections stained with anti-CD21 and anti-IgM\(^a\) or –IgM\(^b\) mAb to permit identification of B6 and B6.NZBc1L germinal centre B cells. A total of 15 PNA\(^+\) germinal centres from 3 different B6 + B6.Thy1\(^a\)IgH\(^a\) controls and 30 germinal centres from 4 different B6.NZBc1L + B6.Thy1\(^a\)IgH\(^a\) chimeric mice were scored for the presence of IgM\(^b\) and IgM\(^a\) expressing cells within the PNA\(^+\) population. In B6 + B6.Thy1\(^a\)IgH\(^a\) controls equivalent proportions of germinal centres contained IgM\(^b\) (11 out of 15) and IgM\(^a\) (11 out of 15) cells. In addition, bright staining with anti-IgM\(^a\) and anti-IgM\(^b\) mAb could be detected on a subset of the CD21\(^{bright}\) cells. This staining was granular in nature suggesting that the anti-IgM\(^a\) and anti-IgM\(^b\) mAbs were binding to immune complexes on the surface of the follicular dendritic cells. These data indicate that in normal mice both IgM\(^b\) and IgM\(^a\) expressing cells are similarly recruited into germinal centres and produce IgM antibodies that can complex with germinal centre antigens. In contrast, there were significantly more IgM\(^b^+\) germinal centres (27 out of 30) than IgM\(^a^+\) germinal centres (8 out of 30) in B6.NZBc1L + B6.Thy1\(^a\)IgH\(^a\) chimeric mice (p < 0.0001 Fisher’s exact test), indicating that B6.NZBc1L B cells are preferentially recruited into autoreactive germinal centres. In
support of this concept and consistent with the significantly elevated levels of IgM\textsuperscript{b}, but not IgM\textsuperscript{a}, autoantibodies in B6.NZBc1L + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} chimeric mice, bright granular staining of CD21\textsuperscript{hi} cells was seen only with anti-IgM\textsuperscript{b} mAb (see Figure 2.3D-F).

Because the majority of PNA\textsuperscript{+} cells within the germinal centres were IgM\textsuperscript{a} and IgM\textsuperscript{b} negative (see Figure 2.3D-F), we stained additional sections with anti-CD21 and anti-IgG2a\textsuperscript{a} or IgG2a\textsuperscript{b} to determine whether the germinal centre B cells had undergone class-switching. As shown in Figures 2.3G-I, the majority of germinal centres (60%) contained large numbers of IgG2a\textsuperscript{b} cells and these were found within both the PNA\textsuperscript{+} and PNA\textsuperscript{-} populations. In contrast, no IgG2a\textsuperscript{a} cells could be seen in any of the germinal centres of B6.NZBc1L + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} chimeric mice.

Taken together, these findings indicate that B6.NZBc1L B cells have an intrinsic functional defect that leads to enhanced recruitment of these cells into autoreactive germinal centres and permits class switching of autoreactive B cells.
FIGURE 2.3 Assessment of “a” and “b” allotype B cells in splenic germinal centres. Spleens from (A) B6 + B6.Thy1^aIgH^a bone marrow or (B) B6.NZBc1L + B6.Thy1^aIgH^a bone marrow chimeric mice were stained with peanut agglutinin (PNA) to delineate germinal centres. Panel (C) shows the PNA staining for the spleen of a representative 4 month old B6.NZBc1L mouse for comparison. Panels D-F, show a germinal centre from a B6.NZBc1L + B6.Thy1^aIgH^a chimeric mouse. All sections were stained with anti-CD21 to reveal follicular dendritic cells within the germinal centre. Shown is one of the few germinal centres with both (E) IgM^a and (F) IgM^b staining cells within the (D) PNA^+ region. Another germinal centre from a B6.NZBc1L + B6.Thy1^aIgH^a chimeric mouse stained with (G) anti-CD21 and PNA to reveal germinal centres. Panel (H) shows staining of the same germinal centre with anti-IgG2a^a and (I) -IgG2a^b. As shown in these panels none of the germinal centres stained with anti-IgG2a^a, whereas germinal centres with abundant IgG2a^b staining cells were seen frequently. Shown in the top right corner of each panel are the stains performed. Magnification; A-C, ×2.5; D-F, ×40; G-I, ×20.
2.4.3- Increased activation of histone-reactive CD4$^+$ T cells in B6.NZBc1L and B6.NZBc1S mice.

B6.NZBc1S and B6.NZBc1L mice produce significantly elevated levels of IgG anti-chromatin antibodies (165). Since histone-reactive T cells have been shown to provide support for IgG anti-chromatin antibody production (357,358), we investigated whether histone-reactive T cells were activated in B6.NZBc1S and B6.NZBc1L mice. To this end, chromosome 1 congenic and control mice were aged to 8 month and their splenic CD4$^+$ T cells purified by negative selection. The purified T cells were then incubated with irradiated T-cell depleted B6 APC together with various concentrations of purified bovine H1, H2A, H2B, H3, H4 or total histones, and T cell activation quantified by measurement of cytokine secretion and proliferation.

As shown in Figure 2.4A, T cells from both B6.NZBc1L and B6.NZBc1S mice demonstrated increased production of IFN-γ as compared to B6 T cells for virtually all of the histones tested. This achieved statistical significance in B6.NZBc1L mice with H1 (p < 0.05) and H3 (p < 0.05) stimulation and in B6.NZBc1S mice with total histone (p < 0.05) and H2A (p < 0.05) stimulation. In contrast, no IL-4 was elaborated following stimulation with any of the histones tested (data not shown). This did not reflect a general inability of these mice to produce IL-4 because IL-4 could be readily detected in the supernatants of wells stimulated with anti-CD3 or ConA, and the levels of these cytokines were comparable in all three strains (data not shown).

In contrast to the findings for cytokine secretion, significant antigen-specific proliferation following stimulation with histones was seen only for B6.NZBc1L mice,
with CD4+ T cells from B6.NZBc1L mice demonstrating significantly increased proliferation in response to stimulation with total histones (p = 0.0005), H2B (p < 0.0001), and H3 (p < 0.05) (Figure 2.4B). T cells from B6.NZBc1L mice were not generally more hyper-proliferative, because these cells proliferated comparably to B6 and B6.NZBc1S cells in response to stimulation with ConA and anti-CD3. Although T cells from 8 month old B6.NZBc1S mice did not proliferate in response to histones, proliferation could be detected with T cells from 12 month old mice (data not shown). This finding suggests that differences between B6.NZBc1S and B6.NZBc1L mice at 8 month of age reflects accelerated kinetics of the response in B6.NZBc1L mice rather than qualitative differences. Taken together the data indicate that the lupus susceptibility locus located within the ~82-106 interval leads to activation of T cells specific for nucleosome histone components and that the susceptibility locus in ~35-82 interval serves to enhance this process.
FIGURE 2.4 Presence of T cells responsive to nucleosome histone components in B6.NZBc1L and B6.NZBc1S mice. Splenic CD4+ T cells, isolated from 8 to 9 month old B6 (▲), B6.NZBc1L (●) and B6.NZBc1S (■) mice, were cultured together with B6 T-cell depleted APC pulsed with 1μg/ml of total histones, H1, H2A, H2B, H3 or H4. (A) Histone-induced IFN-γ production. IFN-γ was measured in the supernatant of cells cultures for 48 hours. Each symbol represents the mean of a triplicate determination from an individual mouse. (B) Histone-induced proliferation. Cells were cultured for 48 hours and pulsed for the last 16 hour with [3H]-thymidine. Uptake of [3H]-thymidine was quantified by a scintillation counter and expressed as mean cpm ± SD of triplicate wells. The stimulation index was calculated by dividing the mean cpm in presence of antigen by the mean cpm in the absence of antigen (i.e. T cells + APCs only). Each symbol represents the determination from an individual mouse. Horizontal lines indicate the mean for each population examined. Mean background proliferation (T cells + APCs only) was similar for B6, for B6.NZBc1L and for B6.NZBc1S.
2.4.4- *Self-reactive B cells drive T cell activation in B6.NZBc1L but not B6.NZBc1S mice.*

We have previously shown that introduction of a non-self reactive Ig transgene recognizing hen egg white lysozyme (Ig Tg) onto the B6.NZBc1L background resulted in normalization of the B cell activation phenotype (165), suggesting that the increased B cell activation in these mice reflects enhanced activation of self-reactive B cells. As a result of this constriction of the B cell repertoire, the proportions of activated (CD69⁺) and memory (CD44hiCD62lo) CD4⁺ T cells in B6.NZBc1L Ig Tg mice were significantly reduced, indicating that autoreactive B cells were amplifying T cell activation. However, the proportion of memory T cells in B6.NZBc1L Ig Tg mice was not normalized to levels seen in B6 Ig Tg mice, but remained significantly elevated at levels comparable to those seen in B6.NZBc1S non-Tg mice. This suggested that the increased activation of T cells observed in B6.NZBc1S mice might be B cell-independent. To address this question the Ig Tg was backcrossed onto the B6.NZBc1S background.

As in our previous study, greater than 95% of B cells in Ig Tg mice were HEL-specific (data not shown) and the proportion of activated B cells was normalized in B6.NZBc1L and B6.NZBc1S Ig Tg mice to levels seen in control B6 Ig Tg mice ([Table 2.2](#)). Consistent with our previous results, the proportions of CD69⁺ and CD44hiCD62Llo CD4⁺ T cells in B6.NZBc1L mice were markedly reduced by introduction of the Ig Tg, but remained elevated as compared to B6 Ig Tg mice, at levels seen in non-Tg B6.NZBc1S mice ([Figure 2.5](#)). In contrast, introduction of the Ig Tg on the B6.NZBc1S background had no impact on T cell activation, with the proportion of memory T cells remaining significantly higher than corresponding B6 Ig Tg controls ([Figure 2.5](#)). These
data indicate that T cell activation in B6.NZBc1L, but not B6.NZBc1S mice, is amplified by the presence of activated self-reactive B cells.
Table II. Normalization of the B cell activation phenotype in B6.NZBc1L and B6.NZBc1S mice by introduction of an Ig transgene

<table>
<thead>
<tr>
<th></th>
<th>NTg</th>
<th></th>
<th>Tg</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>B6</td>
<td>B6.NZBc1L</td>
<td>B6.NZBc1S</td>
<td>B6</td>
</tr>
<tr>
<td></td>
<td>(N = 8)</td>
<td>(N = 4)</td>
<td>(N = 11)</td>
<td>(N = 11)</td>
</tr>
<tr>
<td>% CD69(^+) B220(^+)</td>
<td>6.31 ± 1.30</td>
<td>13.2 ± 2.25 **</td>
<td>7.71 ± 2.57</td>
<td>4.19 ± 2.35</td>
</tr>
<tr>
<td>% B7.1(^+) CD21(^{lo}) B220(^+)</td>
<td>11.67 ± 4.13</td>
<td>28.73 ± 6.84 **</td>
<td>19.25 ± 6.44 *</td>
<td>8.18 ± 3.34</td>
</tr>
<tr>
<td>MFI B7.2 CD21(^{lo}) B220(^+)</td>
<td>43.03 ± 11.38</td>
<td>63.69 ± 7.72 *</td>
<td>32.58 ± 10.10</td>
<td>34.65 ± 9.68</td>
</tr>
<tr>
<td>MFI ICAM-1 CD21(^{lo}) B220(^+)</td>
<td>334.4 ± 73.3</td>
<td>462.5 ± 54.4 *</td>
<td>408.8 ± 192</td>
<td>281.8 ± 98.2</td>
</tr>
</tbody>
</table>

\(^{1}\) Results are mean ± SD. Numbers in brackets denote the number of 4-mo-old female mice examined in each group. Significance level for comparison of B6.NZBc1L and B6.NZBc1S non-transgenic mice with B6 non-transgenic controls or B6.NZBc1L and B6.NZBc1S transgenic mice with B6 transgenic controls, as determined by Mann-Whitney non-parametric test.

\(*\ p < 0.05.\)

\(**\ p < 0.005.\)
FIGURE 2.5 Impact of an Ig transgene on T cell activation in B6.NZBc1L or B6.NZBc1S congenic mice. Freshly isolated splenocytes from 4 month old mice were stained with anti-CD4 together with anti-CD69, or anti-CD62L and –CD44, to determine the proportion of (A) activated CD69+ or (B) memory CD44hiCD62Llo CD4+ T cells in non-Tg (NTg, ▲ = B6, ● = B6.NZBc1L and ■ = B6.NZBc1S) and anti-HEL Ig Tg (Tg, △ = B6, ○ = B6.NZBc1L and □ = B6.NZBc1S) mice. Each symbol represents the determination from an individual mouse, with horizontal lines indicating the mean for each population.
2.4.5- The increased B cell activation in B6.NZBc1L mice is T cell-dependent indicating the presence of a positive feed back loop.

In NZB mice, one of the manifestations of polyclonal B cell activation is increased costimulatory molecule expression on diverse B cell subsets (230). This phenotype is seen in 3-4 week old mice and is CD40L and T cell-independent suggesting that it arises from an intrinsic B cell defect (manuscript in preparation and unpublished observations). In B6.NZBc1L mice, increased costimulatory molecule expression does not develop until approximately 4 month of age and, as outlined above, is most marked in the CD21lo B cell subset. Because of these differences, we were interested in determining whether the B cell activation phenotype in these mice is similarly T cell-independent. We therefore crossed a TCRα chain knockout onto the B6.NZBc1L genetic background and assessed B cell costimulatory molecule expression in 4 to 6 month old mice using flow cytometry.

Comparison of B cell costimulatory molecule expression on the CD21lo B cell population of B6.NZBc1L.TCRα+/− and B6.NZBc1L.TCRα−/− mice revealed that B7.1 (p < 0.0001), and B7.2 (p < 0.001) were significantly reduced in knockout mice (Figure 2.6). Although there was a trend to decreased ICAM-1 expression, this did not achieve statistical significance. Similar findings were observed for CD69 expression in the total B cell population (%B cells CD69+, B6.NZBc1L.TCRα+/− = 8.75 ± 3.26, B6.NZBc1L.TCRα−/− = 6.25 ± 2.57, p < 0.05). Costimulatory molecule expression in the CD21int and CD21hi populations was not increased in B6.NZBc1L.TCRα+/−, as compared to B6.TCRα+/- mice, and there was no impact of the TCR knockout on this expression (data not shown). The proportion of B cells in peripheral B cell subsets as defined by
staining with anti-CD21 and –CD23, was also unaffected by the TCR knockout (data not shown).

Despite decreased costimulatory molecule expression in the CD21lo subset of B6.NZBc1L.TCRα/- mice, B7.1 remained elevated in this subset relative to B6.TCRα/- mice (p < 0.0005). It is likely that this residual B cell activation results from activation by the few residual T cells in these mice. Both B6.TCRα/- and B6.NZBc1L.TCRα/- mice had small populations of T cells that stained with anti-TCR β and γ/δ mAb, and germinal centres could still be seen in these mice (data not shown). Consistent with the presence of residual T cell help in these mice, B6.NZBc1L.TCRα/- mice produced IgG anti-ssDNA antibodies (OD ± SD; B6.NZBc1L.TCRα+/- = 0.525 ± 0.747, B6.NZBc1L.TCRα/- = 0.389 ± 0.258, p = NS) and -chromatin antibodies (OD ± SD; B6.NZBc1L.TCRα+/- = 0.360 ± 0.267, B6.NZBc1L.TCRα/- = 0.374 ± 0.286, p = NS). In keeping with previous reports (359,360), B6.TCRα/- mice also produced IgG anti-ssDNA antibodies (OD ± SD; B6.TCRα+/- = 0.096 ± 0.062, B6.TCRα/- = 0.462 ± 0.492, p = 0.050), and showed a trend to increased IgG anti-chromatin antibody production (OD ± SD; B6.TCRα+/- = 0.095 ± 0.068, B6.TCRα/- = 0.145 ± 0.189, p = NS).
FIGURE 2.6 Costimulatory molecule expression on splenic B cells of TCRα+/+, TCRα+/- and TCRα-/- mice. Freshly isolated splenocytes from 4 to 6 month old TCRα+/+ mice (▲ = B6, ● = B6.NZBc1L), TCRα+/- mice (△ = B6, ○ = B6.NZBc1L) and TCRα-/- mice (◇ = B6, ▽ = B6.NZBc1L) were stained with anti-B220, anti-CD21, and anti-B7.1 or -B7.2 antibodies. The percent of B7.1+ cells and MFI for B7.2 in the CD21loB220+ population are shown. Each symbol represents the determination from an individual mouse, with horizontal lines indicating the mean for each population.
The observation that there was no difference between B6.TCRα-/- and B6.NZBc1L.TCRα-/- mice in the proportions of the residual T cell populations or their activation (as indicated by expression of CD69) suggests that the increased B7.1 expression in B6.NZBc1L.TCRα-/-, as compared to B6.TCRα-/- B cells, reflects an intrinsic B cell defect that affects the response to T cell help (data not shown). Thus, T cells drive the activation of self-reactive B cells in B6.NZBc1L mice, which in turn amplify T cell activation (as demonstrated in Ig Tg mice), resulting in a positive feedback loop and exacerbation of autoimmune disease in B6.NZBc1L mice.

2.5- Discussion

Congenic mice are excellent tools for dissecting the complex genetic basis of murine SLE. To define the functional defects associated with the development of autoimmunity on NZB chromosome 1, we generated mice with short and long chromosome intervals. In our previous study, we provided evidence for at least two genetic loci on chromosome 1. One located within the ~82 to 106 cM interval affecting T cell activation and second within the ~35 to 82 cM interval that appears to affect B cell activation and leads to enhanced autoantibody production, glomerulonephritis, splenomegaly, and T cell activation (165). Here we have demonstrated that the altered T and B cell activation in these mice results from intrinsic T and B cell functional defects and provide insight into the nature and location of these defects together with their role in the development of autoimmunity.

Although our mixed chimeric experiments used B6.NZBc1L bone marrow cells, our findings permit localization of intrinsic B and T cell defects not only to the
B6.NZBc1L interval (~35-106 cM) but also to the B6.NZBc1S interval (~82-106 cM). In B6.NZBc1S mice autoimmunity is characterized by production of IgG anti-ssDNA and – chromatin antibodies. We show that production of these autoantibodies is critically dependent upon the presence of an intrinsic B cell defect. Similarly, the increased proportion of CD4+ memory T cells, a phenotype localized to the B6.NZBc1S interval, is restricted to congenic T cells in B6.NZBc1L + B6.ThylαIgHα mixed chimeric mice. We further characterized this T cell defect, by demonstrating that the abnormal T cell activation in B6.NZBc1S mice is B cell-independent and associated with priming of IFN-γ producing T cells with specificity for nucleosome histone components. Thus, in B6.NZBc1S mice, an intrinsic T cell defect that leads to abnormal activation of histone-reactive T cells and an intrinsic B cell defect that permits the abnormal differentiation of autoreactive B cells into antibody producing cells act together to produce autoimmunity.

Our data also provides insight into how the additional susceptibility allele(s) in B6.NZBc1L mice leads to enhancement of autoimmunity. Using B6.NZBc1L + B6.ThylαIgHα chimeric mice we show that enhanced upregulation of costimulatory molecules, a phenotype that we have localized to the ~35 to 82 interval, reflects an intrinsic B cell defect. We further demonstrate that the upregulation of costimulatory molecules in B6.NZBc1L B cells is T cell-dependent and requires Ig receptor engagement, suggesting that self-reactive B cells in these mice have altered responses to T cell help. In support of this concept we have found that B6.NZBc1L anti-HEL Ig Tg B cells demonstrate enhanced differentiation to antibody producing cells following transfer into B6.NZBc1L soluble HEL recipient mice (Nan Chang and Joan Wither, unpublished observations). The more robust T cell activation in B6.NZBc1L, as compared to
B6.NZBc1S, mice appears to be driven by interactions with these functionally altered self-reactive B cells and it is probable that the enhanced histone-specific proliferative response of B6.NZBc1L T cells is due to the same process.

Despite the presence of activated self-reactive congenic B cells in B6.NZBc1L + B6.Thy1aIgHa mixed chimeric mice, activation of B6 T cells is not elevated above that seen in B6 + B6.Thy1aIgHa mixed chimeric mice. This observation suggests that the ability of B cells to drive T cell activation is dependent upon the presence of an intrinsic T cell defect. Thus, in B6.NZBc1L mice, interactions between at least two independent susceptibility loci lead to a positive feedback loop in which functionally altered T cells activate functionally altered B cells, and vice versa, resulting is amplification of the autoimmune phenotype. This data provides an important experimental confirmation of the role of self-reinforcing B and T cell interactions in the pathogenesis of lupus, as originally proposed by Shlomchik et al (317).

The lack of autoantibodies of B6 origin in B6.NZBc1L + B6.Thy1aIgHa chimeric mice suggests that activation of histone-reactive T cells in these mice is insufficient to break tolerance to chromatin. This finding contrasts with previous work indicating that, anergic chromatin/dsDNA-reactive B cells can become activated to enter germinal centres and differentiate into autoantibody producing cells, if provided with a source of cognate T cell help (361-363). It is possible that T cell help in B6.NZBc1L + B6.Thy1aIgHa chimeric mice differs qualitatively or quantitatively from that seen in these studies. As outlined above, B6.NZBc1L and B6.NZBc1S mice mount a predominant Th1 response to histones, producing IFN-γ but not IL-4. It has been shown that Th1 cells producing high levels of IFN-γ can support anti-chromatin antibody production and
germinal centre formation, whereas T cells with lower levels of IFN-\(\gamma\) provide only limited help for entry into germinal centres and do not support antibody production (364). Our findings for normal B6 B cells in B6.NZBc1L + B6.Thy1\(^a\)IgH\(^a\) chimeric mice are similar to those observed for T cells with low IFN-\(\gamma\) levels, suggesting that the production of IFN-\(\gamma\) may be limiting in these mice. This limited T cell help may be sufficient to provide support only for the functionally abnormal congenic B cells. Alternatively, T\(_{reg}\) cells could inhibit production of autoantibodies by normally tolerant B6 B cells in B6.NZBc1L + B6.Thy1\(^a\)IgH\(^a\) chimeric mice. There is increasing data, from a variety of experimental systems where generation of T\(_{reg}\) cells has been impaired, implicating T\(_{reg}\) cells in the regulation of lupus autoantibody production (359,365). In studies demonstrating a T cell dependent loss of B cell tolerance to chromatin/dsDNA by normal B cells, tolerant B cells were experimentally manipulated to present non-self peptides to T cells, thus evading normal T cell tolerance mechanisms (361,363). In one of these experimental systems, addition of T\(_{reg}\) prevented differentiation of chromatin/dsDNA B cells into antibody forming cells as well as entry into germinal centres (363). In B6.NZBc1L + B6.Thy1\(^a\)IgH\(^a\) chimeric mice, B6 B cells may be more susceptible than congenic B cells to the effects of this T\(_{reg}\)-mediated inhibition.

The preferential ability of congenic B cells to be recruited into autoreactive germinal centres and differentiate into autoantibody forming cells in B6.NZBc1L + B6.Thy1\(^a\)IgH\(^a\) chimeric mice, suggests that these B cells have an intrinsic defect that disturbs tolerance. B cell tolerance to chromatin and dsDNA is maintained by multiple central and peripheral tolerance mechanisms including: clonal deletion, receptor editing, clonal anergy, follicular exclusion, down regulation of the Ig receptor, and germinal
centre tolerance mechanisms (366-369). Our results do not support a clonal deletion and/or receptor editing defect in B6.NZBc1L mice. Upregulation of CD69 in B6.NZBc1L and B6.NZBc1S B cells is dependent upon Ig receptor engagement and interaction with T cells. In B6.NZBc1L + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} chimeric mice, increased proportions of both B6.NZBc1L and B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} B cells express elevated levels of CD69, suggesting that similar proportions of B6 and congenic B cells have engaged self-antigens and received signals from autoreactive T cells. If clonal deletion and/or receptor editing was defective in congenic B cells, then the proportion of self-reactive congenic B cells, and thus CD69+ B cells, should have been increased compared to B6 B cells.

In normal mice dsDNA- and chromatin-reactive B cells that have evaded central deletion or editing are rendered anergic and/or down regulate their Ig receptors (367-369). Although these cells can be recruited into germinal centres, in unmanipulated non-autoimmune mice they fail to differentiate into autoantibody producing cells, undergo class-switching, or develop into memory cells (368). In-vitro, anergic B cells demonstrate impaired responses to Ig receptor engagement (370,371). For example, anergic B cells do not upregulate costimulatory molecules following Ig receptor crosslinking. It has been proposed this reduced expression of costimulatory molecules results in reduced T cell costimulation leading to decreased cytokine production and ineffective T cell help (372). Our results demonstrating differential expression of costimulatory molecules, but not CD69, on congenic, as compared to B6, B cells in B6.NZBc1L + B6.Thy1\textsuperscript{a}IgH\textsuperscript{a} chimeric mice, suggest that self-reactive congenic B cells may be ‘less’ anergic than their B6 counterparts. As a consequence of reduced anergy, congenic B cells may more effectively activate self-reactive T cells leading to enhanced
cytokine production, and support for entry into germinal centres, antibody production, and class switching.

The T cell- and Ig receptor-dependent enhanced expression of costimulatory molecules in B6.NZBc1L mice was most marked in the CD21lo B cell subset. Although transitional, CD5+, and germinal centre B cells, as well as, plasmablasts, are found in the CD21lo B cell subset, analysis of this subset in B6.NZBc1L mice has revealed that the majority of these cells are transitional and germinal centre B cells (Y. H. Cheung and J. Wither, unpublished observations). The increased proportion of CD21lo B cells that we have previously identified in B6.NZBc1L mice is due to an increased number of germinal centre B cells accumulating in this compartment and these cells have high levels of costimulatory molecules (data not shown). It is therefore probable that the increased proportion of congenic CD21lo B cells with high levels of costimulatory molecules in B6.NZBc1L + B6.Thy1aIgHa chimeric mice reflects increased recruitment of these cells into germinal centres. Although we also noted a trend to increased expression of B7.1 on AA4.1+ IgMb, as compared to IgMa, immature B cells in these mice (data not shown).

Several candidate genes have been proposed for the ~82-106 cM interval found in B6.NZBc1S mice. These include: Ifi202, Fcgr2b, and the SLAM/CD2 gene cluster (164,170,181,182). Ifi202, a transcriptional regulator, has been proposed to promote the development of autoimmunity in NZB chromosome 1 congenic mice through impaired B cell apoptosis (164). As outlined above, our data are not consistent with a central B cell deletion defect. Furthermore, although we can readily demonstrate reduced apoptosis of NZB transitional B cells following IgM crosslinking, this process was normal in
B6.NZBc1L mice ((240) and data not shown). Thus, if Ifi202 promotes autoimmunity in NZB mice, it is probable that it does so through some other mechanism.

A promoter polymorphism of the *Fcgr2b* gene, that leads to decreased expression of FcγRIIB on germinal centre B cells, has also been proposed to promote auto-antibody production in NZB mice (181,182). However, recent work indicates that the FcγRIIB receptor has little impact on germinal centre selection mechanisms and instead appears to play an important role in providing a negative feedback signal to germinal centre B cells limiting progression of class-switched B cells to plasma cells (183). Therefore, it is unlikely that the B cell tolerance defect in B6.NZBc1L and B6.NZBc1S mice is due solely the *Fcgr2b* polymorphism in this interval.

Extensive polymorphisms in the SLAM/CD2 gene cluster were identified in B6.*Sle*1 congenic mice, with an introgressed New Zealand White chromosome 1 interval, and proposed as candidate genes for the New Zealand Mixed (NZM) mouse strain (170). NZB mice have the same SLAM/CD2 haplotype and likely share the same T cell signaling abnormality as this strain. It should be noted that B6.*Sle*1 mice have a similar T cell activation phenotype to B6.NZBc1S mice, with a B cell-independent increase in T cell activation and evidence of spontaneous priming of histone-reactive T cells (234,373). However, recent work suggests that in B6.*Sle*1 mice this phenotype is derived from the *Sle1a* and *Sle1c* loci, and not the SLAM/CD2 cluster-containing *Sle1b* locus (374). Thus, B6.NZBc1S mice may share more than one susceptibility alleles with B6.*Sle*1 mice. If this is the case, then the most likely additional susceptibility allele is *Sle1a* because the polymorphism in *Cr2* that has been proposed as a strong candidate gene for *Sle1c* is not found in NZB mice (177). Aberrant activation of B cells with increased IL-6 secretion,
STAT3/SOCS activation, and ras/MAPK activation, has also been described for B6.Sle1ab mice (375). The impact of these signaling abnormalities on B cell tolerance is currently unclear, but our data suggests that it is likely that similar abnormalities are present in B6.NZBc1S mice. Ongoing experiments in the laboratory are seeking to determine which aspects of the B cell tolerance defect that we have described for B6.NZBc1L B cells can be localized to the ~82-106 interval.

Although no candidate genes have been proposed for the ~35-82 interval, this interval contains a number of potential candidates including bcl-2, ship, and several tyrosine-phosphatase genes and regulators of G-protein signaling. Subcongenic mice are currently being generated to more precisely locate the region containing the susceptibility gene within this interval, prior to further investigation of these genes.

The data outlined in this study clearly demonstrate that intrinsic B and T cell defects must act in concert to produce the autoimmune phenotype in B6.NZBc1L and B6.NZBc1S mice. They further indicate that epistatic genetic interactions can occur when functionally abnormal B and T cells interact with each other producing a positive feedback loop. Notably, humans with lupus share many of the features of B6.NZBc1L mice including activation of histone-reactive B cells, increased B cell costimulatory molecule expression and increase recruitment of B cells into germinal centres (376-378). It is therefore tempting to speculate that similar functional abnormalities will be required for the development of human SLE.
Acknowledgments

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CHAPTER 3

Genetic complexity in lupus pathogenesis: autoimmunity in B6.NZBc1 mice reflects interactions between multiple susceptibility loci and a suppressor locus

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3.1- Abstract

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease with a strong and complex genetic basis. To dissect the function of the lupus susceptibility loci on New Zealand Black (NZB) mouse chromosome 1, our lab had originally generated congenic mice with an introgressed homozygous NZB chromosome 1 intervals extending from ~35 or ~82 to 106 cM on the C57BL/6 background. B6.NZBc1(35-106) mice had an increased proportion of activated B and T cells, and made high titre IgG ANAs. When compared with B6.NZBc1(35-106) mice, B6.NZBc1(82–106) mice had significantly less B and T cell activation and lower titres of ANAs, suggesting the presence of at least two lupus susceptibility loci on NZB chromosome 1. Using hematopoietic radiation chimeras with a mixture of B6 and B6.NZBc1(35-106) bone marrow we showed that the increased T and B cell activation in these mice arose from intrinsic B and T cell functional defects. In this study, we have produced and characterized additional subcongenic mouse strains to more precisely localize these susceptibility loci and investigate the mechanisms by which they facilitate development of autoimmunity. Our results demonstrate the presence of at least four lupus susceptibility loci on NZB chromosome 1 between 35 and 106 cM that act coordinately to enhance the autoimmune phenotype. Our findings also suggest the presence of a suppressor locus, located outside the NZB chromosome 1 ~70-100 cM interval that inhibits the production of IgG autoantibodies and development of kidney disease, further demonstrating the complexity of genetic interactions in the development of lupus.
3.2- Introduction

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by the loss of tolerance to self-antigens, particularly nuclear antigens, resulting in the production of anti-nuclear antibodies (ANA) (1-3). These antibodies form immune complexes that deposit in the tissues producing clinical manifestations of disease such as glomerulonephritis, skin rash, and arthritis (4-6). Considerable insight into the immune abnormalities that lead to human SLE has been derived from the study of lupus-prone mouse strains. In particular, the New Zealand black (NZB) mouse and its F1 cross with the New Zealand white mouse (NZB/W) are considered to be excellent models of SLE. NZB mice produce IgG anti-ssDNA, -lymphocyte, and -RBC antibodies that lead to hemolytic anemia and mild glomerulonephritis (GN) (reviewed in (121)). Although these mice do not develop the severe GN that is characteristic of the NZB/W mouse strain, they appear to possess most of the immunologic defects required since replacement of the H-2d MHC locus of NZB mice with H-2bm12 (as in NZB.H-2bm12 congenic mice) is sufficient to produce this phenotype (123).

To facilitate identification of the susceptibility loci that lead to development of autoimmune disease in NZB mice, we have produced congenic mouse strains, in which a homozygous NZB chromosomal interval containing a single or small cluster of susceptibility alleles has been introgressed onto the non-autoimmune C57BL/6 (B6) background. Congenic mice with a NZB chromosome 1 interval extending from 35-106 cM (61.9–190.5 Mb), termed B6.NZBc1(35-106), develop high titre IgG anti-chromatin and –ssDNA antibodies together with mild GN. This is accompanied by cellular phenotypic abnormalities including splenomegaly, increased proportions of activated T
and B cells, and increased numbers of germinal centres (GC) (165). Examination of hematopoietic radiation chimeras with a mixture of B6 and B6.NZBc1(35-106) bone marrow indicated that these phenotypes arose from intrinsic B and T cell functional defects (233). Notably, mice with a shorter NZB chromosomal interval extending from 82-106 cM (Mb; previously denoted B6.NZBc1(85-106)) demonstrated an attenuated phenotype suggesting that there was a second susceptibility locus in the 35-82 cM interval (165).

In this study we have sought to gain further insight into the location and function of the lupus susceptibility alleles on NZB chromosome 1, through creation and investigation of additional subcongenic mouse strains. Our results indicate the presence of at least four susceptibility alleles and a suppressor allele on NZB chromosome 1. The first susceptibility allele is located within the 96-100 cM interval and contains the previously identified Slam, Ifi202, and Fcgr2b candidate gene loci (164,170,181,182). A second susceptibility locus is located in the 88-96 interval that is associated with increased renal disease, and a third locus in the 70-82 interval results in high titre anti-dsDNA antibody production and severe GN with a significant mortality. We further demonstrate the presence of a suppressor locus in the 35-62 cM interval that abrogates this phenotype, and an additional susceptibility allele in the 102-106 cM interval that restores a milder autoimmune phenotype. Functional characterization of T cells in these mice indicates that genetic loci within the 96-100 and 70-82 loci affect T cell function, leading to enhanced production of IFN-γ, whereas the suppressor locus inhibits this production. Taken together the data indicate that there is substantial previously unrecognized genetic complexity in the NZB chromosome 1 35-106 cM interval and that
the autoimmune phenotype in these mice reflects the balance between susceptibility and suppressor alleles that function at least in part through effects on T cell function.
3.3- Methods

3.3.1- Mice

B6 and NZB mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and Harlan-Sprague-Dawley (Blackthorne, England), respectively, and subsequently bred in our facility. Congenic mice were produced by backcrossing mice with a NZB chromosome 1 interval onto the B6 background, using the speed congenic technique. Mice were typed at each successive generation using polymorphic microsatellite markers that discriminate between NZB and B6 DNA, spaced at ~ 20 cM intervals throughout the genome except for regions containing lupus susceptibility genes where more densely spaced markers were used. Fully backcrossed mice were obtained in 6 generations and then intercrossed to produce congenic mice that were homozygous for the NZB intervals. The original chromosome 1 congenic mice that was generated (B6.NZBc1(35-106)) contains an introgressed NZB interval extending from between rs13475886 (30.1cM or 61.2Mb) and rs3158129 (30.1 cM or 61.9Mb) to between D1Mit223 (106.3cM or 190.5Mb) and D1Mit17 (106.3cM or 191.4Mb). The subcongenic mice examined in this study were derived from B6.NZBc1(35-106) congenic mice by backcrossing with B6 mice and selecting offspring with informative crossovers (Figure 3.1). These were then intercrossed to produce homozygous mice. All mice were housed in microisolators in the animal facility at the Toronto Western Hospital (Toronto, Canada) and were specific-pathogen free. The mice that were examined in this study were all female.
FIGURE 3.1 Genetic maps of chromosome 1 congenic lines used in our studies. B6.NZBc1(43-85), B6.NZBc1(96-100), B6.NZBc1(88-100), B6.NZBc1(70-100), B6.NZBc1(35-102) and B6.NZBc1(35-106) mouse strains are shown. Thick and thin lines denote NZB and B6 regions, respectively. Dashed lines indicate regions of undefined origin. Polymorphic microsatellite markers and single nucleotide polymorphisms (SNP) were used to discriminate between NZB and B6 DNA at the termini of the regions according to the NCBI m37 mouse genome assembly (<www.ensembl.org>). Markers are shown to the right of each chromosome representation, with the position of these markers shown to the left. A scale is shown on the far left of the figure. Genes shown to the left of scale are the possible candidate genes located within 96-100 cM interval.
3.3.2- Flow cytometry analysis

RBC-depleted splenocytes ($5 \times 10^5$) were incubated with 10 µg/ml mouse IgG (Sigma-Aldrich, St Louis, MO) for 15 min to block Fc receptors and stained with various combinations of directly-conjugated mAbs. Following washing, allophycocyanin-conjugated streptavidin (BD PharMingen, San Diego, CA) was used to reveal biotin-conjugated Ab staining. Dead cells were excluded by staining with 0.6 µg/ml propidium iodide (PI; Sigma-Aldrich). Flow cytometry of the stained cells was performed using a dual laser FACScalibur (BD Biosciences, Mountain View, CA) and analyzed using Cell Quest Pro software (BD Biosciences). Live cells were gated on the basis of PI exclusion and scattering characteristics with 10,000 events being acquired for each sample. The following directly conjugated mAbs were purchased from BD PharMingen: biotin conjugated anti-CD11c (N418), -CD11b (Mac1), -CD4 (L3T4), -CD8 (53-6.7), and -CD62L (MEL-14); PE conjugated anti-B7.1 (16-10A1), -B7.2 (GL1), -CD3 (145-2C11), -CD69 (H1.2F3), -CD44 (IM7), -NK1.1 (PK136), and -CD4 (H129.19); and FITC conjugated anti-CD3 (145-2C11), -CD4 (L3T4), -CD8 (53-6.7), -CD21/CD35 (7G6), -CD25 (7D4), -B220 (RA3-6B2), -CD11c (HL3) and -CD11b (M1/70). FITC anti-CD62L was purchased from Cedarlane (Hornby, Ontario, Canada). All isotype controls, with the exception of hamster IgG controls (BD Pharmingen), were purchased from Cedarlane.

3.3.3- CD4\(^+\) T cell isolation

CD4\(^+\) T cells were isolated using the Dynal Mouse CD4 Negative Isolation Kit (114.15D, Invitrogen Dynal AS, Oslo, Norway). In brief, single-cell suspensions of RBC-depleted splenocytes ($100 \times 10^6$ cells per ml) were suspended in 10% FBS/PBS and
incubated with a cocktail of biotinylated antibodies on ice for 20 minutes. Excess antibodies were removed by washing and the cells resuspended in 10% FBS/PBS at a concentration of 100 x 10^6 cells per ml. Streptavidin-conjugated Dynabeads were washed with PBS to remove azide, resuspended and added to the cell suspension and incubated at room temperature for 15 minutes. CD4^+ T cells were then purified by negative selection with a magnet and resuspended in 0.5% NMS/RPMI1640. The resultant cell population contained more than 90% CD4^+ T cells.

3.3.4- Anti-CD3 T cell proliferation

Purified CD4^+ T cells were loaded with 5 μM CFSE (Molecular Probes, Eugene, OR) by incubating 5 x 10^7 cells per ml with CFSE for 10 minutes at 37°C. Labeling was quenched with one wash of ice-cold 10% FBS/RPMI1640 and one wash of ice-cold RPMI1640. CFSE labeled CD4^+ T cells were then resuspended in 0.5%NMS/RPMI1640 and cultured at 2.5 x 10^5 cells per well for 72 hours at 37°C in 96 well flat bottom plates with 0.1 μg/ml to 10 μg/ml of immobilized anti-CD3 antibody (Cedarlane) in the presence or absence of 1 μg/ml of anti-CD28 antibody (BD Pharmingen). Proliferation was measured by CFSE dilution using flow cytometry.

3.3.5- Cytokine assays

Cytokine levels in tissue culture supernatants were measured at 72 hr. Anti-IL-2, -IL-4 and -IFN-γ capture antibodies, biotinylated-anti-IL-2, -IL-4 and -IFN-γ detection antibodies, and recombinant IL-2, IL-4 and IFN-γ were purchased from BD Pharmingen. Assays were performed as per the manufacturer’s recommendations. The concentration
of cytokine in each supernatant was calculated from a standard curve of absorbance versus concentration of recombinant cytokine.

3.3.6- Measurement of Ab production

Serum levels of IgM and IgG anti-chromatin, -dsDNA and -ssDNA Abs were measured by ELISA. ssDNA was prepared by boiling dsDNA (isolated from calf thymus DNA) for 10 minutes and quick cooling on ice for 2 minutes. H1-stripped chromatin was prepared from chicken RBC, as described previously (356). ELISA plates were coated overnight with chromatin (8 µg/ml), dsDNA (40 µg/ml) or ssDNA (20 µg/ml) diluted in PBS at 4°C. The plates were then washed with 0.05% Tween 20/PBS, and blocked with 2% BSA/PBS for 1h at room temperature. After further washing serum samples, diluted 1/100 in PBS/BSA/Tween 20, were added. Bound antibodies were detected using alkaline phosphatase-conjugated anti-IgG and anti-IgM (Caltag, Burlingame, CA) as secondary reagents. For measurement of total IgM and IgG, plates were coated with goat anti-mouse IgM and IgG (Jackson ImmunoResearch), respectively, and the serum was diluted 1/1000. The amount of bound IgM or IgG was calculated from a standard curve using purified class-specific controls, and the Ab concentration was calculated from a plot of concentration versus absorbance.

Anti-RBC Ab production was assessed by flow cytometry. Heparinized blood was washed twice in 2% FBS in PBS and stained with FITC anti-IgM (Caltag Laboratories), -IgG (Caltag Laboratories) or -hamster IgG as an isotype control (BD Pharmingen).
3.3.7- Immunofluorescence Staining of Tissue Sections.

Spleens were snap frozen in OCT compound (Sakura Finetek, Torrance, CA) at the time of sacrifice. Cryostat sections (5 μm) were fixed in acetone, washed with PBS, and blocked with 5% normal goat serum/PBS. Sections were stained with biotinylated-PNA (Sigma-Aldrich) and FITC anti-B220 to detect germinal centres. Biotin staining was revealed using rhodamine-conjugated streptavidin as a secondary reagent (Molecular Probes, Eugene, OR). Stained sections were mounted with Mowiol (Calbiochem, La Jolla, CA) and tissue fluorescence visualized using a Zeiss Axioplan 2 imaging microscope (Oberkochen, Germany). Digital images were obtained using the manufacturer’s imaging system.

3.3.8- Grading of kidney sections

Kidneys were fixed in formalin, paraffin embedded, sectioned (3 μm), and stained with periodic acid-Schiff (PAS). Grading was performed by a renal pathologist (G. Lajoie) who was blinded as to the strain of origin of the tissue section. The grading scale used for light microscopy was as follows: grade 0, normal glomeruli; grade 1, mesangial expansion and/or proliferation; grade 2, focal segmental (endocapillary) proliferative glomerulonephritis; grade 3, diffuse (endocapillary) proliferative glomerulonephritis; and grade 4, diffuse proliferative glomerulonephritis with crescents. Glomerular staining of kidney sections stained with FITC anti-IgG were graded by immunofluorescence microscopy. Sections with no or only trace deposits were graded as 0; those with mesangial deposits, grade 1; those with mesangial and segmental capillary wall deposits,
grade 2; those with diffuse mesangial and capillary wall deposits, grade 3; and those with crescents, grade 4.

3.3.9- **Statistics**

Statistical significance of comparisons between groups of mice was determined using the Mann-Whitney non-parametric test except when comparing renal scores between experimental groups where \( \chi^2 \) test for trend was used.
3.4- Results

3.4.1- Characterization of the autoimmune phenotype in subcongenic mouse strains

We have previously shown that congenic mice with a NZB chromosome 1 interval extending from 35-106 cM made significantly higher titres of autoantibodies and had more severe GN than mice with a 82-106 cM interval, suggesting the presence of at least two susceptibility loci, one within the 35-82 interval (locus 1) and the other within the 82-106 (locus 2) interval (165). Based upon the absence of an autoimmune phenotype in mice with a NZB 100-109 cM interval, we hypothesized that the second susceptibility locus was located within the 82-100 interval. These results were consistent with our previous mapping study which demonstrated a broad area of linkage to several B cell activation phenotypes with peaks at ~ 70cM and 96 cM (126). To further investigate the location and function of these susceptibility loci, additional subcongenic mice were produced (see Figure 3.1) including mice with an NZB interval extending from 43-85 cM, containing only locus 1; mice with 96-100 cM and 88-100 cM intervals, containing locus 2; and mice with a 70-100 cM interval, which was proposed to contain both loci.

Cohorts of mice from each subcongenic strain were then aged to 4 or 8 month and autoantibody production, GN, and mortality were examined. Figure 3.2 shows the results for anti-nuclear antibody production at 4 month. Both B6.NZBc1(96-100) and B6.NZBc1(88-100) mice produced similar levels of anti-nuclear antibodies suggesting the presence of a single susceptibility locus in the 96-100 interval. Consistent with the presence of an additional susceptibility locus in the NZB 70-82 cM interval, B6.NZBc1(70-100) mice made significantly higher titres of auto-antibodies than
B6.NZBc1(88-100) and B6.NZBc1(96-100) mice. Notably, mice with an NZB 43-85 cM interval did not produce IgG or IgM autoantibodies, suggesting that the lupus susceptibility locus in the 43-85 cM interval is insufficient to induce a breach of tolerance to nuclear antigens.
FIGURE 3.2 Production of anti-nuclear antibody in subcongenic mice. Autoantibody levels in the serum of 4 month old B6 (●), B6.NZBc1(43-85) (○), B6.NZBc1(96-100) (▲), B6.NZBc1(88-100) (△), B6.NZBc1(70-100) (▼), B6.NZBc1(35-102) (▽) and B6.NZBc1(35-106) (◆) congenic mice. An ELISA was used to assay for the presence of (A) IgM or (B) IgG anti-ssDNA, anti-dsDNA and anti-chromatin. Each symbol represents the determination of an individual mouse. Horizontal lines represent the mean. The p values for significant differences between the congenic mouse strains are shown, *p<0.05, **p<0.005, ***p<0.0005 determined by Mann-Whitney non-parametric test.
B6.NZBc1(35-102) mice were also produced to confirm that genes in the telomeric region of NZB chromosome 1 were not involved in the generation of the autoimmune phenotype. Surprisingly, mice with a 35-102 interval, produced significantly lower levels of IgG autoantibodies when compared with B6.NZBc1(35-106) mice (Figure 3.2), indicating the presence of an additional susceptibility locus in B6.NZBc1(35-106) mice. Since the centromeric crossover for these two mouse strains is in the same 1.8 Mb interval between rs13475886 (30.1cM or 61.2Mb) and rs3158129 (30.1cM or 61.9Mb), it is likely that this locus is located in the 102-106 cM interval.

Notably, B6.NZBc1(35-102) mice also had significantly lower levels of IgG autoantibodies as compared to B6.NZBc1(70-100) mice (Figure 3.2). This finding suggests the presence of a suppressor locus that is located within the NZB 35-70 or 100-102 interval.

3.4.2- Increased mortality and severity of kidney disease observed in B6.NZBc1(70-100) mice

While the subcongenic cohorts were being aged it was noted that a number of B6.NZBc1(70-100) mice died beginning at ~ 3 month of age. Figure 3.3A shows the survival results for the subcongenic mouse strains examined. With the exception of the B6.NZBc1(70-100) strain, death of mice during the first 8 month of life was rare. In contrast, ~ 40% of B6.NZBc1(70-100) mice died during this period. To gain insight into the immune mechanisms leading to this increased mortality, cohorts of B6.NZBc1(70-100) mice were bled every 2 weeks and monitored for development of autoantibodies, hemolytic anemia, and GN. None of the mice produced significant titres
of IgG anti-RBC antibodies or developed anemia. In contrast, mice that died showed an increase in blood urea nitrogen (BUN, an indicator of severe renal disease) immediately prior to death that was accompanied by increased production of IgG anti-dsDNA antibodies (Figure 3.3B). This suggested that the deaths observed in B6.NZBc1(70-100) mice were associated with severe renal failure. Consistent with this possibility, examination of kidney sections from B6.NZBc1(70-100) mice that were pre-morbid demonstrated the presence of diffuse proliferative GN. Notably, high-grade proteinuria was absent in pre-morbid mice. Instead, pre-morbid demonstrated hematuria, suggesting the presence of a nephritic rather than nephrotic picture.
FIGURE 3.3 Increased Mortality in B6.NZBc1(70-100) mice and association of B6.NZBc1(70-100) mice mortality with the development of IgG anti-dsDNA Ab and kidney disease. (A) Survival Curve of B6 (●, N=26), B6.NZBc1(43-85) (○, N=29), B6.NZBc1(96-100) (▲, N=21), B6.NZBc1(88-100) (△, N=12), B6.NZBc1(70-100) (▼, N=19), B6.NZBc1(35-102) (▽, N=7) and B6.NZBc1(35-106) (◆, N=32) congenic mice. Mice were tracked and mortality was recorded up to 8 month of age. The p values for significant differences between B6 and other congenic mouse strains are shown, **p<0.005, N.S. = not significant as determined by log-rank test of survival. (B) B6 and B6.NZBc1(70-100) was monitored for the production of serum IgG anti-dsDNA Ab and the level of blood urea nitrogen (BUN) beginning at 2 month of age until 7 to 7.5 month old (or until the mice died or sacrificed for experiments). An ELISA was used to assay for the presence IgG anti-dsDNA and the BUN level. Each symbol represents the determination of an individual mouse. Circle denotes mice that died at the age shown. A
total of 8 B6.NZBc1(70-100) mice and 4 B6 controls were monitored in the cohort study shown.

To further characterize the nature and severity of kidney disease in the various subcongenic mouse strains, the kidneys of mice sacrificed at 8 month of age were examined for IgG deposition and light microscopic changes, with the severity of the changes being graded using a four-point scale as outlined in Materials and Methods. The kidneys from 8 month old B6.NZBc1(88-100), B6.NZBc1(70-100) and B6.NZBc1(35-106) mice demonstrated significantly increased IgG deposition and more severe renal disease than B6 controls (Figure 3.4). Despite the death of ~40% of the B6.NZBc1(70-100) mice with the most severe kidney disease by this time point, the majority of remaining mice still demonstrated severe changes with diffuse proliferative glomerulonephritis. In general, the severity of light microscopic changes paralleled the extent of IgG deposition, suggesting that differences in the amount and specificity of IgG autoantibodies rather than the renal response to injury dictated the severity of renal involvement. This concept is further supported by the observation that the mice with the most severe kidney involvement all produced significant titres of IgG anti-dsDNA antibodies, which has been closely correlated with the severity of renal disease in previous studies. Notably, significant renal abnormalities were not seen in B6.NZBc1(96-100) mice, suggesting the presence of an additional locus within the NZB 88-96 cM chromosome 1 interval that controls the development of renal disease in these mice. Consistent with the presence of a suppressor locus in B6.NZBc1(35-102) mice that abrogates IgG anti-nuclear antibody production, kidney disease was significantly attenuated in these mice as compared to that seen in B6.NZBc1(70-100) mice.
FIGURE 3.4 Renal involvement in 8 month old subcongenic mice. (A) Immunofluorescence scores of frozen kidney sections stained with anti-IgG. Sections were graded as follows: grade 0, no or only trace deposits; grade 1, mesangial deposits; grade 2, mesangial and segmental capillary wall deposits; grade 3, diffuse mesangial and capillary wall deposits; grade 4, crescents. (B) Glomerular scores of kidneys fixed in formalin, paraffin embedded, sectioned, and stained with PAS. Sections were graded as: grade 0, normal glomeruli; grade 1, mesangial expansion and/or proliferation; grade 2, focal segmental proliferative glomerulonephritis; grade 3, diffuse proliferative glomerulonephritis; and grade 4, diffuse proliferative glomerulonephritis with crescents.
Each symbol represents the determination from an individual mouse. Horizontal lines indicate the mean for each population examined.

3.4.3- Cellular abnormalities in the subcongenic mouse strains

To gain further insight into the immune mechanisms through which the various loci on NZB chromosome 1 act to facilitate or impair development of autoimmune disease, we performed an extensive cellular characterization of the various subcongenic mice at 4 month of age. All of the subcongenic mouse strains demonstrated increased spleen weights and cellularity as compared to B6 mice. We have previously shown that B6.NZBc1(35-106) mice have altered B cell selection with increased proportions of CD21\textsuperscript{intermediate (int)}CD23\textsuperscript{+}, predominantly follicular, B cells and decreased proportions of CD21\textsuperscript{high(hi)}CD21\textsuperscript{-} marginal zone B cells (233). These findings were restricted to B6.NZBc1(88-100) and B6.NZBc1(70-100) mice, suggesting that genetic locus (loci) that produces this phenotype maps to the 88-100 interval.

B6.NZBc1(35-106) mice also demonstrated increased B cell activation, reflected by increased levels of expression of B7.1, B7.2, ICAM-1, and CD69, predominantly in their CD21\textsuperscript{low(lo)} B cell compartment. As shown in Table 3.1 and Figure 3.5, increased B cell activation was seen in all of the subcongenic mouse strains, but was most marked in B6.NZBc1(70-100) mice. Since increased B cell activation was seen in both B6.NZBc1(96-100) and B6.NZBc1(43-85) mice, it is likely that the loci in these mice contribute additively to the increased B cell activation observed in B6.NZBc1(70-100) mice. Notably, B cell activation was significantly reduced in B6.NZBc1(35-102) mice and largely restored in B6.NZBc1(35-106) mice, indicating that the additional genetic loci in these mice inhibit and augment B cell activation, respectively.
**TABLE 3.1**

*Table 1. Comparison of splenic phenotype in B6.NZBc1 subcongenic strains*.

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<td>Numbers of 4 months old mice examined in each group unless otherwise indicated in brackets.</td>
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<td>N = 12</td>
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<td>N = 24</td>
<td>N = 14</td>
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<td>spleen weight (mg)</td>
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<td>122.0 ± 22.64c</td>
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<td>150.5 ± 46.08c</td>
<td>165.4 ± 60.70c</td>
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<td># splenocytes (x 10⁶)</td>
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<td>% B220⁺</td>
<td>54.23 ± 6.74</td>
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<td>61.66 ± 5.49</td>
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<td>% B7.1⁺</td>
<td>17.66 ± 5.06</td>
<td>15.37 ± 3.05</td>
<td>19.84 ± 3.79</td>
<td>19.26 ± 3.97</td>
<td>22.02 ± 5.36b</td>
<td>18.48 ± 4.45</td>
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<td>MFI B7.2</td>
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<td>MFI ICAM-1</td>
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<td>% CD21⁺⁺CD23⁻</td>
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¹ Results are mean ± SD. Significance level for comparison of various NZB chromosome 1 subcongenic strains with B6 control, as determined by Mann-Whitney non-parametric test

² p < 0.05

³ p < 0.005

⁴ Numbers of 4 months old mice examined in each group unless otherwise indicated in brackets.
Increased proportions of recently activated (CD69+) and memory/effector (CD44hiCD62Llo) T cells are also seen in B6.NZBc1(35-106) mice. Of the subcongenic strains examined, B6.NZBc1(96-100), B6.NZBc1(88-100), and B6.NZBc1(70-100) mice all had roughly equivalently increased levels of recently activated CD4+ T cells suggesting that the genetic locus that produces this phenotype is located within the 96-100 interval. These mice also had increased proportions of memory/effector T cells, consistent with chronically increased activation of their CD4+ T cell subset and similar but less pronounced findings were seen for their CD8+ T cell subset. Although the proportion of recently activated CD4+ T cells in B6.NZBc1(43-85) mice was not significantly elevated, these mice had increased proportions of memory/effector CD4+ and CD8+ cells, suggesting that their T cells were also being chronically activated but to a lesser extent. In contrast, B6.NZBc1(35-102) mice demonstrated a marked reduction in T cell activation when compared with B6.NZBc1(35-106) and B6.NZBc1(70-100) mice, even though the proportion of memory T cells in B6.NZBc1(35-102) remain significantly increased relative to B6 controls. This finding suggests that the suppressor locus inhibits T cell activation.
FIGURE 3.5 Activation phenotype of splenic CD21lo B cells from subcongenic mice. Freshly isolated splenocytes from 4 month old B6 (●), B6.NZBc1(43-85) (○), B6.NZBc1(96-100) (▲), B6.NZBc1(88-100) (△), B6.NZBc1(70-100) (▼), B6.NZBc1(35-102) (▽), and B6.NZBc1(35-106) (◆) congenic mice were stained with anti-B220, and anti-CD21 with either anti-B7.1, -B7.2 or -ICAM-1 antibody. The percent of B7.1+, and the mean fluorescent intensity (MFI) of B7.2 or ICAM-1 within the B220+CD21lo B cell population are shown. The p values for significant differences between the congenic mouse strains and controls are shown, *p<0.05, **p<0.005, ***p<0.0005 as determined by Mann-Whitney non-parametric test.
To further explore the nature of T cell help for autoantibody production in subcongenic mice we quantified the number and size of germinal centres. We have previously shown that B6.NZBc1(35-106) mice have an increased number and size of germinal centres, as compared to control B6. As shown in Figure 3.6, all of the subcongenic mouse strains examined had an increased number of splenic germinal centres, which was most marked in the B6.NZBc1(88-100), B6.NZBc1(70-100), and B6.NZBc1(35-106) mouse strains. In general, the number and size of the germinal centres observed in these mouse strains correlated with each other and paralleled the extent of chronic T cell activation observed by flow cytometry (see Table 3.2 and Figure 3.6).


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<tr>
<td><strong>% CD4&lt;sup&gt;a&lt;/sup&gt;</strong></td>
<td>19.64 ± 2.75</td>
<td>18.70 ± 2.83</td>
<td>19.22 ± 4.41</td>
<td>19.55 ± 3.38</td>
<td>22.62 ± 3.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.25 ± 2.33</td>
<td>22.15 ± 2.72&lt;sup&gt;a&lt;/sup&gt;</td>
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<td><strong>% CD69&lt;sup&gt;a&lt;/sup&gt;</strong></td>
<td>22.13 ± 4.30</td>
<td>26.05 ± 6.05</td>
<td>32.26 ± 8.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.14 ± 8.75&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.85 ± 5.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.37 ± 7.12</td>
<td>32.55 ± 7.19&lt;sup&gt;c&lt;/sup&gt;</td>
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<td><strong>% CD4&lt;sup&gt;a&lt;/sup&gt;CD62L&lt;sup&gt;b&lt;/sup&gt; (Memory)</strong></td>
<td>24.81 ± 4.65</td>
<td>38.96 ± 9.64&lt;sup&gt;c&lt;/sup&gt;</td>
<td>39.12 ± 10.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33.18 ± 10.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>39.40 ± 9.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.92 ± 7.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.16 ± 8.13&lt;sup&gt;c&lt;/sup&gt;</td>
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<td><strong>% CD4&lt;sup&gt;a&lt;/sup&gt;CD62L&lt;sup&gt;b&lt;/sup&gt; (Naïve)</strong></td>
<td>48.54 ± 11.15</td>
<td>32.75 ± 12.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.87 ± 10.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.82 ± 14.62</td>
<td>34.38 ± 13.97&lt;sup&gt;c&lt;/sup&gt;</td>
<td>46.36 ± 11.75</td>
<td>33.43 ± 15.66&lt;sup&gt;e&lt;/sup&gt;</td>
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<td><strong>% CD8&lt;sup&gt;a&lt;/sup&gt;</strong></td>
<td>13.24 ± 1.95</td>
<td>11.52 ± 1.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.11 ± 2.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.66 ± 2.21&lt;sup&gt;f&lt;/sup&gt;</td>
<td>11.04 ± 2.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.71 ± 1.65 (12)</td>
<td>11.28 ± 2.11&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td><strong>% CD4&lt;sup&gt;a&lt;/sup&gt;CD62L&lt;sup&gt;b&lt;/sup&gt; (Memory)</strong></td>
<td>7.41 ± 2.82</td>
<td>14.11 ± 8.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.38 ± 6.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.57 ± 3.99</td>
<td>10.40 ± 3.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.95 ± 2.41 (12)</td>
<td>12.67 ± 5.69&lt;sup&gt;a&lt;/sup&gt;</td>
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<td><strong>% CD4&lt;sup&gt;a&lt;/sup&gt;CD62L&lt;sup&gt;b&lt;/sup&gt; (Naïve)</strong></td>
<td>47.70 ± 10.05</td>
<td>40.27 ± 13.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.44 ± 12.30</td>
<td>52.95 ± 12.03</td>
<td>48.46 ± 18.26</td>
<td>53.54 ± 11.55 (12)</td>
<td>46.08 ± 13.76</td>
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<td><strong>% CD4&lt;sup&gt;a&lt;/sup&gt;CD25&lt;sup&gt;c&lt;/sup&gt;CD62L&lt;sup&gt;b&lt;/sup&gt;</strong></td>
<td>1.04 ± 0.19 (17)</td>
<td>0.52 ± 0.11&lt;sup&gt;f&lt;/sup&gt; (9)</td>
<td>0.73 ± 0.34&lt;sup&gt;f&lt;/sup&gt; (8)</td>
<td>0.77 ± 0.21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.77 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.79 ± 0.37 (6)</td>
<td>0.83 ± 0.26 (8)</td>
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<td><strong>CD4+/CD8+ ratio</strong></td>
<td>1.49 ± 0.16</td>
<td>1.65 ± 0.33</td>
<td>1.78 ± 0.36&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.87 ± 0.26&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.12 ± 0.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.53 ± 0.27 (12)</td>
<td>1.99 ± 0.25&lt;sup&gt;c&lt;/sup&gt;</td>
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1 Results are mean ± SD. Significance level for comparison of various NZB chromosome 1 subcongenic strains with B6 control, as determined by Mann-Whitney non-parametric test.

<sup>a</sup> p < 0.05

<sup>b</sup> p < 0.005

<sup>c</sup> p < 0.0005

<sup>f</sup> Numbers of 4 months old mice examined in each group unless otherwise indicated in brackets.
Figure 3.6: Immunofluorescence microscopy and quantitation of splenic germinal centres. (A) Spleens from 4 month old B6.NZBc1(43-85), B6.NZBc1(96-100), B6.NZBc1(88-100), B6.NZBc1(70-100), B6.NZBc1(35-102) and B6.NZBc1(35-106) congenic mice were stained for dual-color immunofluorescence. Green and red colors depict staining for B220 and PNA, respectively. Note the marked increase in the size of germinal centres in all NZB chromosome 1 congenic mice except B6.NZBc1(43-85). Magnification = 10X. (B-C) Scatterplot showing the number of germinal centres per B220⁺ lymphoid follicle (B) and size (in mm²) of 20 randomly selected germinal centre (C) s in B6 (●), B6.NZBc1(43-85) (○), B6.NZBc1(96-100) (▲), B6.NZBc1(88-100) (△), B6.NZBc1(70-100) (▼), B6.NZBc1(35-102) (▼) and B6.NZBc1(35-106) (■) splenic sections. Horizontal lines indicate the mean for each population examined.
A deficiency of regulatory T cells has been implicated in the increased T cell activation seen in mice with a chromosome 1 interval from the New Zealand Mixed (NZM) mouse strain. We therefore questioned whether the increased activation seen in NZB chromosome 1 subcongenic mice, could reflect a deficiency in this population. To this end, we quantified the proportion of CD4+CD25+CD62L+ splenocytes, the population that was thought to represent T regulatory (Treg) cells examined in the previous studies (379). As shown in Table 3.2, all of the mouse strains had reduced proportions of these cells as compared to B6 mice, which achieved statistical significance for four of the mouse strains. Similar findings were also observed for 8 month old mice, although the results were somewhat more variable (%CD4+CD25+CD62L+: B6 = 0.82% ± 0.35, n = 23; B6.NZBc1(43-85) = 0.40% ± 0.19, n = 22, p < 0.0001; B6.NZBc1(96-100) = 0.73 ± 0.06, n = 4, p = N.S.; B6.NZBc1(88-100) = 0.43 ± 0.15, n = 10, p = 0.0008; B6.NZBc1(70-100) = 1.29 ± 0.51, n = 7, p = 0.0273; B6.NZBc1(35-102) = 0.54 ± 0.20, n = 6, p = N.S.; B6.NZBc1(35-106) = 0.37 ± 0.15, n = 10, p = 0.0001). As recent work suggests that FoxP3+ is a better marker of Treg cells and that CD25 correlates closely with regulatory cell function in this population (380), we also quantified the proportion of CD4+CD25+FoxP3+ splenocytes (Figure 3.7). With the exception of B6.NZBc1(43-85) and B6.NZBc1(96-100) mice the proportion of these cells in 8 month old mice was similar to that observed in B6 mice (%CD4+CD25+FoxP3+ cells: B6 = 0.921 ± 0.36, n = 27; B6.NZBc1(43-85) = 0.71 ± 0.31, n = 21, p = 0.045; B6.NZBc1(96-100) = 0.38 ± 0.11, n = 8, p = 0.0004; B6.NZBc1(88-100) = 0.90 ± 0.23, n = 10, p = N.S.; B6.NZBc1(70-100) = 1.18 ± 0.24, n = 6, p = N.S.; B6.NZBc1(35-102) = 0.87 ± 0.43, n = 7, p = N.S.; B6.NZBc1(35-106) = 0.90 ± 0.29, n = 12, p = N.S.). Although we have more
limited data for 4 month old mice, at this age the proportion of CD4⁺CD25⁺FoxP3⁺ cells in B6.NZBc1(43-85) and B6.NZBc1(96-100) mice was similar to B6 controls (%CD4⁺CD25⁺FoxP3⁺ cells: B6 = 1.28 ± 0.11, n = 3; B6.NZBc1(43-85) = 1.06 ± 0.23, n = 4, p = N.S.; B6.NZBc1(96-100) = 1.12 ± 0.23, n = 3, p = N.S.). These findings indicate that the proportion of CD4⁺CD25⁺CD62Lhi cells does not accurately reflect the proportion of functional Treg cells, which appears to be normal in younger NZB chromosome 1 congenic mice at the initiation of autoimmune disease. Thus, it is unlikely that the increased activation observed in the various 4 month old subcongenic mouse strains results from altered Treg cell function.
FIGURE 3.7 Foxp3 expression profile of splenic CD4+ T cells from subcongenic mice. Freshly isolated splenocytes from 8 month old B6, B6.NZBc1(43-85), B6.NZBc1(96-100), B6.NZBc1(88-100), B6.NZBc1(70-100), B6.NZBc1(35-102) and B6.NZBc1(35-106) congenic mice were stained with anti-CD4 and anti-CD25. After permeabilization, the splenocytes were further stained intracellularly with anti-Foxp3 antibody. Numbers shown in each histogram showing the percentage of Foxp3+ within the CD4+, CD4+CD25− or CD4+CD25+ T cell populations.
3.4.4- Altered threshold for T cell activation in subcongenic mice

We have previously shown that the increased activation of T cells in B6.NZBc1(35-106) results from an intrinsic T cell defect (233). This finding suggested that the T cells of these mice have altered function. To assess this possibility and to determine the location of the genetic locus (loci) that contribute to this functional abnormality, CD4+ T cells were purified from young pre-autoimmune 8 to 12 week old mice and stimulated with immobilized anti-CD3 *in-vitro*. At this age, no significant differences in the proportion of recently activated (CD69+) and memory/effector (CD44hiCD62Llo) T cells were observed between the mouse strains. As shown in Figure 3.8A, CD4+ T cells from all subcongenic strains except B6.NZBc1(43-85) demonstrated increased proliferation at suboptimal concentrations of anti-CD3 antibody. Representative histograms of CFSE dilution profile for each mouse strain examined were shown in Figure 3.8B. Notably, proliferation was similarly increased in all of these strains, indicating that the increased proliferation maps to the 96-100 interval and that the suppressor locus does not function by inhibiting T cell proliferation. In contrast, all of the subcongeneric mouse strains examined demonstrated increased IFN-γ production with anti-CD3 mAb. At low concentrations of anti-CD3 mAb this was restricted to B6.NZBc1(70-100) T cells. These findings suggest that loci within the 43-85 and 96-100 cM intervals are associated with increased IFN-γ production and that they act additively to further enhance IFN-γ production in B6.NZBc1(70-100) mice. IFN-γ production was similarly reduced in B6.NZBc1(35-102) and B6.NZBc1(35-106) mice, suggesting that the suppressor locus in the 35-102 interval impairs generation of this cytokine and that the susceptibility locus in the 102-106 interval does not restore this production.
FIGURE 3.8A CD4⁺ T cells from subcongenic mice are hyperresponsive to anti-CD3 stimulation. Splenic CD4⁺ T cells were isolated from 8-12-week-old B6 (N=17), B6.NZBc1(43-85) (N=7), B6.NZBc1(96-100) (N=11), B6.NZBc1(70-100) (N=6), B6.NZBc1(35-102) (N=5) and B6.NZBc1(35-106) (N=8) by negative selection. Splenic CD4⁺ T cells were then labeled with carboxyfluorescein succinimidyl ester (CFSE) and stimulated with various concentrations of plate bound anti-CD3 (0.1μg/ml to 10μg/ml) for 72 hours. Cell proliferation was measured using CFSE dilution. ELISA assays were performed to measure the amount of IFN-γ produced by CD4⁺ T cells after 72-hr of culture. The p values for significant differences between B6 and other subcongenic mouse strains are shown, *p<0.05, **p<0.005, ***p<0.0005 as determined by one-way ANOVA followed by Dunnett’s Multiple Comparison Test.
FIGURE 3.8B CFSE dilution profile of splenic CD4⁺ T cells from subcongenic mice upon anti-CD3 crosslinking. Splenic CD4⁺ T cells were isolated from 8-12-week-old B6, B6.NZBc1(43-85), B6.NZBc1(96-100), B6.NZBc1(70-100), B6.NZBc1(35-102) and B6.NZBc1(35-106) by negative selection. Splenic CD4⁺ T cells were then labeled with carboxyfluorescein succinimidyl ester (CFSE) and stimulated with various concentrations of plate bound anti-CD3 (0.1ug/ml to 10ug/ml) for 72 hours. Representative histograms showing CFSE dilution of CD4⁺ T cells upon activation by 1ug/ml of anti-CD3 are shown. Number in each histogram plot represent the proportion of CFSE<sup>low</sup> cells of the representative experiment, after subtracting from the background.
3.5- Discussion

The current study sought to further localize the lupus susceptibility loci on NZB chromosome 1 and to gain insight into the immune mechanisms by which they promote autoantibody production and renal disease. Through generation of multiple subcongenic mice with overlapping chromosomal intervals, we demonstrate considerable genetic complexity in the immunopathogenesis of lupus in NZB chromosome 1 congenic mice, with at least 4 lupus susceptibility loci and a suppressor locus. While our previous mapping study demonstrated a broad area of linkage, indicating the possibility of more than one genetic locus in this interval (126), this extent of genetic complexity was not anticipated. Thus, the NZB chromosome 1 interval joins a growing number of congenic intervals that based upon mapping studies were thought to contain a single susceptibility locus, but upon further dissection using subcongenic mice were found to contain multiple susceptibility loci (163,176,202). These findings are consistent with the concept that the original mapping studies were underpowered to detect individual genetic loci, that in general have relatively small effects and therefore only detected regions where multiple loci interacted additively or multiplicatively to produce a stronger signal.

Our results indicate the presence of at least one lupus susceptibility locus located within the 96-100cM (172.8-181.7Mb) interval of NZB chromosome 1. Genetic polymorphism(s) in this interval lead to a relatively mild autoimmune phenotype with production of anti-nuclear antibodies in the absence of significant renal disease. This region overlaps with the Nba2 and Sle1b loci that were identified in NZB and New Zealand Mixed (NZM) 2410 mouse strains (153,163), respectively, and contains the previously proposed candidate genes slam, ifi202, and fcgr2b (164,170,181,182). The
slam locus encodes a family of adhesion/costimulatory molecules that regulate the activation threshold and type of response for many cells involved in the immune system ((381-383) and reviewed in (384)). NZB, NZM2410, and a variety of other autoimmune mouse strains share the same slam allele, which differs from that of B6 mice (170).

Although there are polymorphisms in many of the slam family members within this locus, recent experiments suggest that it is the polymorphism in Ly108 that alters immune function. Expression of Ly108.1, an alternatively spliced isoform of Ly108, is increased in mice with the autoimmune associated allele, whereas expression of Ly108.2 is decreased (168). These two splice variants differ in their cytoplasmic domain, with the Ly108.1 variant demonstrating an enhanced ability to trigger SAP-dependent tyrosine phosphorylation in T cells (173). Stimulation of Ly108 with an anti-Ly108 mAb has been shown to enhance Th1 cytokine release in human T cells (385). Thus, the increased proliferation and IFNγ secretion by B6.NZBc1(96-100) T cells observed following anti-CD3 cross-linking could arise from this polymorphism.

Although ifi202 and fcgr2b have also been proposed as candidate genes in the NZB 96-100 interval, a recent study examining another series of subcongenic mice with NZB chromosome 1 intervals centering around this interval demonstrated that the presence or absence of the NZB ifi202 allele had little impact on autoimmunity (180). This group also concluded that the fcgr2b locus augments the autoimmune phenotype, since a shorter interval containing the slam locus alone had a milder phenotype than a longer interval containing the slam and fcgr2b loci. However, the autoantibody profile of the mice with the shorter interval containing just the slam locus is similar to that observed for our B6.NZBc1(96-100) mice that contain the fcgr2b locus, whereas that of the longer
interval is similar to our B6.NZBc1(88-100) mice (notably both intervals have similar centromeric crossovers). These observations suggest that the second susceptibility allele in the congenic mice with the longer intervals is unlikely to be the NZB fcgr2b allele, which appears to have little impact on the autoimmune phenotype.

The susceptibility locus located in the 88-96 cM interval (170.3-172.8Mb) leads to increased numbers and sizes of germinal centres, and promotes renal disease. These findings suggest that this locus impacts on qualitative aspects of T cell help. This interval overlaps with the Sle1a susceptibility locus, previously identified in NZM2410 mice. B6.Sle1a mice have several T cell abnormalities including increased T cell activation, reduced numbers and function of Treg cells, and increased CD4+ T cell support for IgG anti-chromatin production by B cells (163,374). Although the predicted function of the NZB susceptibility locus in the 88-96 cM interval is consistent with that of Sle1a, the proportion of CD4+ FoxP3+ CD25+ Treg cells in B6.NZBc1(88-100) mice is normal (see results). We selected this set of markers to quantify Tregs because previous work in autoimmune mouse models suggests that the proportion of CD4+FoxP3+ cells does not accurately reflect Treg function (386), as with age there is an increased proportion of CD4+Foxp3+CD25- cells that are non-functional (387). Consistent with this, the proportion of CD4+FoxP3+ cells was increased in 8 month old B6.NZBc1(88-100) mice (data not shown). Given that the proportion of CD4+Foxp3+ cells is reduced in B6.Sle1a mice, the susceptibility locus in NZB mice appears to be distinct from Sle1a.

A third susceptibility locus is located in the 70-88 cM interval (127.5-170.3Mb) of NZB mice. Addition of this locus to those in the 88-100 cM interval resulted in increased B cell activation, autoantibody titres, immunoglobulin deposition in the kidney,
severity of renal disease, and mortality. It is likely that this locus is centromeric to ~162 Mb, because we previously showed that B6.NZBc1(35-106) mice have a more pronounced phenotype than B6.NZBc1(82-106) mice (165). This region corresponds to the area of peak linkage for IgG anti-ssDNA Ab production and increased expression of ICAM-1 and CD44 on B cells in our previous mapping study (126). It also overlaps with a NZB region identified by Kono et al. that is linked to anti-chromatin Ab production and splenomegaly in a (NZB x NZW)F2 cross (156). By itself (see B6.NZBc1(43-85) mice) this locus appears to be insufficient to breach B cell tolerance to nuclear antigens but does lead to an increase in memory/effector T cells and germinal centres that are small in size. These findings, together with the observation that this locus is associated with increased IFN-γ production following anti-CD3 stimulation, suggest that this locus predominantly affects T cell function.

Autoimmune disease is markedly attenuated in B6.NZBc1(35-102) mice as compared to B6.NZBc1(70-100) mice. It is unlikely that this difference reflects the absence of one or more susceptibility loci in B6.NZBc1(35-102) mice, because extensive genotyping of this interval revealed NZB homozygosity with a largest distance between markers of 4.4 Mb. It is also unlikely that this difference results from genetic differences on other chromosomes that may have been missed by using a speed congenic approach. Several different transgenes and knockouts have been crossed onto the B6.NZBc1(70-100) background, with homozygous wild type mice always retaining the severe autoimmune phenotype. In addition, both the B6.NZBc1(35-102) and B6.NZBc1(35-106) mouse strains demonstrate an attenuated autoimmune phenotype. Although both these mouse strains originated from the same original cross, they were backcrossed
independently to B6 a number of generations prior to intercrossing to produce homozygous mice, arguing that they are unlikely to share a suppressor locus that is not on chromosome 1. It is likely that the suppressor locus is located within the 35-70 interval, since we have recently generated an additional subcongenic mouse line, B6.NZBc1(62-102) from the B6.NZBc1(35-102) mouse line, whose cellular and serologic profile parallels that of B6.NZBc1(70-100) mice (Landolt-Marticorena et al, unpublished observation). Comparison of immunologic function in B6.NZBc1(70-100) and B6.NZBc1(35-102) mice, suggests that the suppressor locus, predominantly affects T cell function. B6.NZBc1(35-102) retained the ability to produce IgM anti-nuclear autoantibodies, suggesting that the breach of B cell tolerance to nuclear antigens remains. However, IgG anti-nuclear and nephrophilic autoantibody production and the number of germinal centres were markedly attenuated, indicating a relative lack of T cell help. This occurred despite the observation that their T cells retained increased IFNγ production and increased proliferation in response to anti-CD3 crosslinking.

Comparison of B6.NZBc1(35-106) and B6.NZBc1(35-102) mice, demonstrates the presence of another susceptibility allele. It is unlikely that this susceptibility allele is located at the centromeric end of the 35-106 cM interval, because we have narrowed the crossover to the same 0.7 Mb interval for both of these mouse strains, which does not contain any genes known to have immunologic function. It is therefore likely that the 102-106 cM (187.7-191.4 Mb) interval contains this susceptibility allele. This interval excludes Sle1c, a NZM susceptibility allele, found on the telomeric end of chromosome 1, but contains Tgfb2 an attractive candidate gene (163). This susceptibility allele appears to be unable to breach B cell tolerance to nuclear antigens on its own, since we have
previously shown that B6.NZBc1(100-109) mice do not produce anti-nuclear antibodies (165). Nevertheless, these mice had elevated levels of MHC on their B cells and showed a trend to increased expression of CD69 on their T cells, suggesting that they may have some underlying cellular abnormalities as compared to B6 mice.

In summary, we have identified several novel susceptibility loci and an intriguing suppressor locus on NZB chromosome 1. Further characterization of these loci and the immune mechanisms by which they interact to facilitate or inhibit autoimmunity should provide new insights into the pathogenesis of lupus.
CHAPTER 4

Epistatic Interactions between Lupus Susceptibility Loci on New Zealand Black Chromosomes 1 and 13 Lead to Marked Expansion of Dendritic Cell Populations but do not Exacerbate Autoimmunity

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Running Title: Uncoupling of dendritic cell expansion and disease severity in bicongenic mice

Keywords: Rodent, Dendritic cells, Systemic Lupus Erythematosus, Cytokines
4.1- Abstract

Genetic loci on New Zealand Black (NZB) chromosomes 1 and 13 play a significant role in the development of lupus-like autoimmune disease and have been linked to the altered B cell phenotype in these mice. We have previously shown that C57BL/6 (B6) congenic mice with homozygous NZB chromosome 1 (B6.NZBc1) or 13 (B6.NZBc13) intervals produce anti-nuclear Ab and develop mild glomerulonephritis. Both mouse strains also demonstrated increased B cell activation, and for B6.NZBc13 mice altered B cell selection, recapitulating many of the NZB B cell phenotypic abnormalities, but to a lesser extent. In this study we sought to determine whether these two intervals were sufficient to fully reproduce the NZB B cell phenotype, through production of bicongenic B6.NZBc1c13 mice. Surprisingly, the B cell phenotype in bicongenic mice remained similar to that observed in the parental strains. However, several novel phenotypes developed including: marked plasmacytoid and myeloid dendritic cell expansion, and elevated levels of BAFF and IgA production. Despite these changes, only minor increases in anti-DNA Ab production and T cell activation were seen, which had no impact on the severity of glomerulonephritis. Furthermore, IFN-α levels remained low, especially in older mice where production of this cytokine appeared to be suppressed. The data indicate that expansion of plasmacytoid dendritic cells and the presence of anti-DNA Ab need not be associated with increases in IFN-α production, revealing additional complexity in the regulation of this cytokine and autoimmunity in systemic lupus erythematosus.
4.2- Introduction

Systemic lupus erythematosus (SLE)\(^3\) is a multisystem autoimmune disease of unknown etiology that mainly affects women of childbearing age. One of the hallmarks of this condition is the loss of tolerance to self-antigens, particularly nuclear Ag, leading to production of anti-nuclear antibodies (ANA) and formation of immune complexes (1). Deposition of these complexes in the glomeruli, skin, joints, and other organs induces tissue damage resulting in the manifestations of disease including; glomerulonephritis, skin rash, and arthritis (349).

The New Zealand Black (NZB) mouse and its F\(_1\) cross with the New Zealand White mouse (NZB/W) are considered to be excellent models of SLE and characterization of the immune defects in these mice has led to a number of fundamental insights into the human disease. In NZB mice, autoimmune disease is characterized by production of IgG anti-ssDNA, -lymphocyte, and -RBC Ab resulting in hemolytic anemia and mild glomerulonephritis (reviewed in (121)). NZB/W mice develop in addition high affinity IgG anti-dsDNA/chromatin auto-Ab that lead to a severe immune complex-mediated glomerulonephritis beginning around 5-7 month of age (139). Although NZB mice do not develop nephritis, they appear to possess most of the immunologic defects required. When the H-2\(^d\) MHC locus of NZB mice is replaced by H-2\(^{bm12}\) (as in NZB.H-2\(^{bm12}\) congeneric mice) anti-dsDNA auto-Ab are produced and rapidly progressive glomerulonephritis ensues (123).

B cell abnormalities are a prominent feature of the immunologic derangement in NZB mice and include: 1) IgM hypergammaglobulinemia; 2) increased numbers of IgM auto-Ab-producing cells; 3) increased proportions of activated B cells with elevated
levels of co-stimulatory molecules; 4) expanded populations of activated B cell subsets such as germinal centre and plasmablasts; and 5) altered proportions of peripheral B cell populations with a reduced proportion of follicular and increased proportion of marginal zone (MZ) and B1a B cells (126,230). We have previously mapped the increased expression of co-stimulatory molecules to NZB chromosome 1 and 13 regions that overlapped with susceptibility loci linked to auto-Ab production and glomerulonephritis (126).

To further explore the immune defects leading to this altered B cell activation and their association with autoimmunity, congenic mice were produced with homozygous NZB chromosome 1 (B6.NZBc1) or 13 intervals (B6.NZBc13) introgressed onto a B6 background (165,212). Both mouse strains demonstrated increased B cell activation and produced ANAs; however, the nature of the B cell phenotypes and auto-Ab produced differed. While B6.NZBc13 mice had increased proportions of MZ and B1a cells, recapitulating the phenotype seen in NZB mice, the B1a splenic population was not expanded in B6.NZBc1 mice and the proportion of MZ B cells was decreased. In addition, B6.NZBc1 mice produced IgG anti-ssDNA, -chromatin and -histone Ab, whereas the IgG response was more focused upon chromatin in B6.NZBc13 mice.

In this study we produced B6.NZBc1c13 bicongenic mice to investigate whether these two loci are sufficient to fully recapitulate the NZB B cell phenotype and lead to enhanced autoimmunity. Surprisingly, while the B cell phenotype in bicongenic mice remained similar to that observed in the parental strains, several novel phenotypes developed. These included marked expansion of splenic myeloid and plasmacytoid dendritic cell (DC) populations, IgA auto-Ab production and elevated BAFF levels.
Despite development of these phenotypes, only minor increases in auto-Ab production and T cell activation were seen, which had no impact on the severity of glomerulonephritis. Furthermore, despite increases in the number of splenic plasmacytoid DC (pDC), splenic IFN-α levels remained low and production of IFN-α appeared to be suppressed in older mice. The findings indicate that genetic interactions between lupus susceptibility loci produce phenotypes that cannot always be predicted based upon their effects in isolation and suggest additional complexity in the regulation of IFN-α production beyond the presence of nuclear Ag-containing immune complexes and pDC.
4.3- Materials and Methods

4.3.1- Mice

B6 and NZB mice were purchased from the Taconic Farms (Germantown, NY) and Harlan-Sprague-Dawley (Blackthorne, England), respectively, and subsequently bred in our facility. Congenic mice were produced by separately backcrossing NZB chromosome 1 and 13 intervals onto the B6 background, using the speed congenic technique (148). Mice were genotyped at each successive generation using polymorphic microsatellite markers that discriminate between NZB and B6 DNA, spaced at ~ 20 cM intervals throughout the genome, except for regions containing lupus susceptibility genes where more densely spaced markers were used. Fully backcrossed mice were produced within 6 or 7 generations, for chromosome 1 and 13 intervals respectively, and then intercrossed to produce congenic mice that were homozygous for the NZB intervals. For NZB chromosome 1 congenic mice (previously called B6.NZBc1(35-106) but here denoted as B6.NZBc1 for simplicity) the NZB interval extends from between D1Mit161 (59.3Mb) and D1Mit303 (63.0Mb) to between D1Mit223 (190.5Mb) and D1Mit210 (192.1Mb). NZB chromosome 13 congenic mice (B6.NZBc13) have an NZB interval extending from between D13Mit117 (37.7Mb) and D13Mit318 (61.0Mb) to between D13Mit77 (117.7Mb) and D13Mit78 (119.6Mb). B6.NZBc1c13 bicongenic mice were produced by intercrossing B6.NZBc1 and B6.NZBc13 mice and selecting for homozygous NZB chromosome 1 and 13 intervals in successive crosses. The mice were housed in microisolators in the animal facility at the Toronto Western Hospital (Toronto, Canada) and were specific-pathogen free. All of the mice that were examined in this
study were female. The animal protocols used in this study were reviewed and approved by the University Health Network Animal Care Committee.

4.3.2- Flow cytometry analysis

RBC-depleted splenocytes (5 x 10^5) were incubated with 10 μg/ml mouse IgG (Sigma-Aldrich, St Louis, MO) for 15 min to block Fc receptors and stained with various combinations of directly-conjugated mAbs. Following washing, allophycocyanin-conjugated streptavidin (BD Biosciences, San Diego, CA) was used to reveal biotin-conjugated Ab staining. Dead cells were excluded by staining with 0.6 μg/ml propidium iodide (PI; Sigma-Aldrich). Flow cytometry of the stained cells was performed using a dual laser FACSCalibur (BD Biosciences, Mountain View, CA) and analyzed using Cell Quest Pro (BD Biosciences) software. Live cells were gated on the basis of PI exclusion and scattering characteristics, with 10,000 or 25,000 events being acquired for each sample. The following directly conjugated mAbs were purchased from BD Biosciences: biotin conjugated anti-CD11c (N418), -CD11b (Mac1), -CD4 (L3T4), -CD8 (53-6.7), and -CD62L (MEL-14); PerCP conjugated anti-B220 (30-F11); PE conjugated anti-B7.1 (16-10A1), -B7.2 (GL1), -CD3 (145-2C11), -CD23 (B3B4), -CD69 (H1.2F3), -CD44 (IM7), -ICAM-1 (3E2), -NK1.1 (PK136), and -CD4 (H129.19); and FITC conjugated anti-CD3 (145-2C11), -CD4 (L3T4), -CD8 (53-6.7), -CD21/CD35 (7G6), -CD25 (7D4), -B220 (RA3-6B2), -CD11c (N418) and -CD11b (Mac-1). FITC PDCA-1 (Miltenyi Biotec) was a generous gift from Dr. Eleanor Fish. FITC anti-CD62L was purchased from Cedarlane (Hornby, Ontario, Canada). All isotype controls, with the exception of hamster IgG controls (BD Biosciences), were purchased from Cedarlane.
4.3.3- Measurement of Ab production

Serum levels of IgM, IgG, and IgA anti-chromatin, -dsDNA and -ssDNA Ab were measured by ELISA. ssDNA was prepared by boiling dsDNA (isolated from calf thymus DNA) for 10 min and quick cooling on ice for 2 minutes. H1-stripped chromatin was prepared from chicken RBC, as described previously (356). ELISA plates were coated overnight with chromatin (8 μg/ml), dsDNA (40 μg/ml) or ssDNA (20 μg/ml) diluted in PBS at 4°C. The plates were then washed with 0.05% Tween 20/PBS, and blocked with 2% BSA/PBS for 1h at room temperature. After further washing, serum samples, diluted 1/100 in 2% BSA/Tween 20/PBS, were added. Bound Ab were detected using alkaline phosphatase-conjugated anti-IgG, -IgM or -IgA Ab (Caltag, Burlingame, CA) as secondary reagents. For measurement of total IgM, IgG, and IgA, plates were coated with goat anti-mouse IgM or IgG (Jackson ImmunoResearch, West Grove, PA, USA) or rat anti-mouse IgA (BD Biosciences) respectively, and the serum was diluted 1/1000. The amount of bound IgM, IgG or IgA was calculated from a standard curve using purified class-specific controls, and the Ab concentration was calculated from a plot of concentration versus absorbance.

4.3.4- APC isolation

Single-cell suspensions of splenocytes were isolated from 2 to 3 month old B6 mice by pressing through a nylon mesh. RBCs were removed by lysis in Gey’s solution. The cell suspension was then incubated with purified anti-Thy1.2 (HO-13-4) and anti-CD4 (GK1.5) mAb followed by guinea pig complement (Cedarlane) to remove T cells.
Following lysis, the resultant cell population (<1% CD4+ T cell contamination) was resuspended in RPMI 1640 containing 0.5% normal mouse serum (NMS).

4.3.5- CD4+ T cell isolation

CD4+ T cells were isolated using the Dynal Mouse CD4 Negative Isolation Kit (114.15D, Invitrogen Dynal AS, Oslo, Norway). In brief, single-cell suspensions of RBC-depleted splenocytes (1 x 10^8 cells per ml) were suspended in 10% FBS/PBS and incubated with a cocktail of biotinylated Ab on ice for 20 min. Excess Ab was removed by washing and the cells resuspended in 10% FBS/PBS at a concentration of 1 x 10^8 cells per ml. Streptavidin-conjugated Dynabeads were washed with PBS to remove azide, resuspended, added to the cell suspension, and incubated at room temperature for 15 min. CD4+ T cells were then purified by negative selection with a magnet and resuspended in 0.5% NMS/RPMI1640. The resultant cell population contained 85% to 95% CD4+ T cells.

4.3.6- T cell proliferation and cytokine assays

T cell-depleted B6 splenic APCs were irradiated with 2000 rads and then incubated for 1 h at 37°C with media alone or containing 1 μg/ml total bovine histones, H1, H2A, H2B, H3, or H4 (Roche, Quebec, Canada). Excess antigen was removed by washing with PBS and the cells resuspended in 0.5% NMS/RPMI1640. Antigen-pulsed APCs were cultured at 5x10^5 cells per well together with purified CD4+ T cells (5 x 10^5 per well) for 48 h at 37°C in 96 well flat bottom plates. Anti-CD3 (5 μg/ml) was added to control wells at the time of plating. Proliferation was measured by [³H]-thymidine
incorporation at 48 h after a 16 h pulse with 1 μCi/well. Uptake of \(^{3}H\)-thymidine was quantified by a scintillation counter and expressed as mean cpm ± SD of triplicate wells. For each Ag condition, a stimulation index was calculated by dividing the mean cpm in the presence of Ag by the mean cpm in the absence of Ag.

IL-4 and IFN-γ levels in tissue culture supernatants were measured at 48 h. Anti-IL4 and -IFN-γ capture Ab, biotinylated-anti-IL4 and -IFN-γ detection Ab, and recombinant IL-4 and IFN-γ were purchased from BD Biosciences. Assays were performed as per the manufacturer’s recommendations. The concentration of cytokine in each supernatant was calculated from a standard curve of absorbance versus concentration of recombinant cytokine.

4.3.7 - TLR stimulation

B cell proliferation in response to TLR stimulation was measured by co-culturing 5 x 10\(^5\) RBC-depleted splenocytes that were stained with 5 μM CFSE together with media alone (0.5%NMS/RPMI1640) or 2 μM Imiquimod R837, 50 μg/ml poly I:C, 250 nM ODN1826 control, 250 nM ODN1826, 250 nM ODN2216 control, 250 nM ODN2216 (all purchased from InvivoGen, San Diego, CA) or 5 μg/ml LPS (Sigma) for 72 h in 96-well plates. The cells were then stained with allophycocyanin-conjugated anti-B220 and the proportion of B cells undergoing at least one cell division quantified by flow cytometry, gating on the B220\(^+\) population. To assess IFN-α, IL-10 and TNF-α production following TLR stimulation, RBC-depleted splenocytes (2 x 10\(^6\) per well) were stimulated in 96-well plates with media alone (0.5%NMS/RPMI1640), 250nM ODN1826 control and 250nM ODN1826, or 250nM ODN2216 control and 250nM ODN 2216 for
48 h. Cytokine levels in tissue culture supernatants were measured by ELISA using commercially available kits as follows: IFN-α, PBL Biomedical Laboratories (Piscataway, NJ); IL-10, SABiosciences Corporation (Frederick, MD); and TNF-α, BD Biosciences or eBioscience (San Diego, CA). Assays were performed as per the manufacturer’s recommendations with the concentration of cytokine calculated from a standard curve of absorbance versus concentration of recombinant cytokine.

4.3.8- Immunofluorescence Staining of Tissue Sections.

Spleens were snap frozen in OCT compound (Sakura Finetek, Torrance, CA) at the time of sacrifice. Cryostat sections (5 μm) were fixed in acetone, washed with PBS, and blocked with 5% normal goat serum/PBS or 5% fetal bovine serum/PBS when goat IgG anti-TNF-α (R&D Systems, Minneapolis, MN) was used. Sections were stained with biotinylated anti-CD11b and FITC anti-CD11c, or biotinylated anti-B220 and FITC anti-CD11c, to detect myeloid and plasmacytoid DC, respectively. BAFF or TNF-α production was assessed by staining in tandem with rabbit IgG anti-BAFF (Sigma) followed by AMCA-conjugated goat anti-rabbit IgG Ab (Jackson ImmunoResearch) or goat IgG anti-TNF-α (R&D Systems, Minneapolis, MN) followed by AMCA-conjugated bovine anti-goat IgG (H+L) Ab (Jackson ImmunoResearch). Biotin staining was revealed using rhodamine-conjugated streptavidin as a secondary reagent (Molecular Probes, Eugene, OR). Stained sections were mounted with Mowiol (Calbiochem, La Jolla, CA) and tissue fluorescence visualized using a Zeiss Axioplan 2 imaging microscope (Oberkochen, Germany). Digital images were obtained using the manufacturer’s imaging system.
4.3.9- **Grading of kidney sections**

Kidneys were fixed in formalin, paraffin embedded, sectioned (3 µm), and stained with periodic acid-Schiff. Grading was performed by a renal pathologist (G. Lajoie) who was blinded as to the strain of origin of the tissue section. The grading scale used for light microscopy was as follows: grade 0, normal glomeruli; grade 1, mesangial expansion and/or proliferation; grade 2, focal segmental (endocapillary) proliferative glomerulonephritis; grade 3, diffuse (endocapillary) proliferative glomerulonephritis; and grade 4, diffuse proliferative glomerulonephritis with crescents. Glomerular staining of kidney sections stained with FITC anti-IgG was graded by immunofluorescence microscopy. Sections with no or only trace deposits were graded as 0; those with mesangial deposits, grade 1; those with mesangial and segmental capillary wall deposits, grade 2; those with diffuse mesangial and capillary wall deposits, grade 3; and those with crescents, grade 4.

4.3.10- **Measurement of mRNA expression**

RNA was purified from splenocytes and bone marrow cells of 8 month old mice using the RNeasy Mini Kit (Qiagen, Basel, Switzerland), treated with DNaseI (Invitrogen, Canada), and converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Forster City, CA), according to the manufacturer’s instructions. Quantitative real-time PCR was performed with SYBR Green Master Mix on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using default cycling conditions. Primer sequences were designed to span exon-to-exon and were as follows: β-actin forward, 5’-TTGCTGACGGATGCAAG-3’, β-actin
reverse, 5’-GTACTTGCGCTCAGGAGGAG-3’; 

baff forward, 5’-TTCCATGGCTTCTCAGCTTT-3’, baff reverse, 5’-CGTCCCCAAAGACGTGTACT-3’;

mifα forward, 5’-GCCACCACGCTCTTCTGTCT-3’; mifα reverse, 5’-TCTGGGCCATAGAAGTGAGA-3’;

pkr forward, 5’-TGAGCGCCCCCATCT-3’, pkr reverse, 5’-TATGCCAAAAGCCAGAGTCCTT-3’;

2’-5’ oas forward, 5’-TGAGCGCCCCCATCT-3’, 2’-5’ oas reverse, 5’-CATGACCCCCGGTCATCAAAGG-3’;

ifn-α4 forward, 5’-CTTGTCTGCTACTTGGGATGCAA-3’, ifn-α4 reverse, 5’-AGGAGGTTCTGCTACACACAA-3’; and ifn-β1 forward, 5’-TGACGGAGAGATGCAGAAGAG-3’, ifn-β1 reverse 5’-CACCCAGTGCTGGAGAAATT-3’. Gene expression was analyzed using the relative standard curve method and was normalized to β-actin expression.

4.3.11 - Statistics

Statistical significance of comparisons between groups of mice was determined using the Mann-Whitney non-parametric test with the exception of comparisons between kidney tissue section grades where Fisher’s exact test was used.
4.4- Results

4.4.1- Loci on NZB chromosome 1 and 13 are insufficient to recapitulate the B cell phenotypic abnormalities in NZB mice.

NZB mice have altered proportions of peripheral B cell subsets in their spleen, with reduced proportions of follicular B cells and increased proportions of MZ B cells (230). These changes are most prominent in younger mice. Indeed, by 8 month of age the progressive immunologic derangement in NZB mice results in a reduced proportion of MZ B cells as compared to B6 mice (Table 4.1). As shown in our previous studies (212), 8 month old B6.NZBc13 mice have B cell phenotypic changes similar to 4 month old NZB mice, with reduced proportions of follicular (CD21\text{intermediate(int)}CD23^+) and increased proportions of MZ (CD21\text{high(hi)}CD23^+) B cells. Surprisingly, these changes were not more pronounced in bicongenic mice. Instead, B6.NZBc1c13 mice had reduced proportions of MZ B cells similar to B6.NZBc1 mice. This was not due to an age associated loss of the MZ B cell population, because 4 month old bicongenic mice demonstrated a similar reduction in their MZ B cell population (%CD21^{\text{hi}}CD23^- cells, B6 = 5.64% ± 1.23, n = 15; B6.NZBc1 = 3.98% ± 1.84, n = 9, p = 0.0342; B6.NZBc13 = 9.76% ± 1.47, n = 2, p = N.D.; B6.NZBc1c13 = 4.38% ± 1.90, n = 11, p = 0.0430; NZB = 9.46% ± 3.93, n = 5, p = 0.0088, all p values as compared to B6 mice; p value for B6.NZBc1c13 as compared to NZB = 0.0092). Similar findings were observed for the splenic B1a cell population, where the proportion of cells in 8 month old bicongenic mice was similar to that observed in B6.NZBc1 mice and was significantly reduced as compared to both B6.NZBc13 and NZB mice (%CD21^{\text{low(lo)}}CD5^+ of B220^+ cells, B6 =
1.48% ± 0.44, n = 9; B6.NZBc1 = 2.98% ± 0.74, n = 10, p = 0.0005; B6.NZBc13 = 5.73% ± 1.44, n = 5, p = 0.0033; B6.NZBc1c13 = 2.93% ± 1.61, n = 8, p = 0.0011; NZB = 5.80% ± 1.04, n = 6, p = 0.0018, all p values as compared to B6; p value for B6.NZBc1c13 as compared to NZB = 0.0019). Thus, the distribution of B cells in bicongenic mice appears to be driven predominantly by genetic loci on NZB chromosome 1, and does not recapitulate that seen in NZB mice.

We have previously published that B cell activation, as measured by up-regulation of CD80, CD86, and ICAM-1, is less marked in B6.NZBc1 or B6.NZBc13 mice, than NZB mice (165,212). At 8 month of age, the levels of these activation markers were similar in bicongenic mice to those seen in parental congenic mouse strains, and for CD80 remained significantly less than those seen in NZB mice (Table 4.1). Similar findings were observed for 4 month old mice, however the differences between B6.NZBc1c13 and NZB mice were more marked (%B7.1 cells, B6.NZBc1c13 = 19.06% ± 5.84, n = 13; NZB = 34.04% ± 9.92, n = 5, p = 0.0058; MFI B7.2, B6.NZBc1c13 = 41.28 ± 19.08, n = 3; NZB = 71.20 ± 32.08, n = 5, p = 0.0385; MFI ICAM-1, B6.NZBc1c13 = 324.3 ± 103.6, n = 13; NZB = 418.9 ± 51.71, n = 5, p = 0.0385). Taken together, the data indicate that genetic loci on NZB chromosomes 1 and 13 are insufficient to recapitulate the altered B cell phenotype in NZB mice.
**TABLE 4.1**

*Table I. Comparison of the splenic B220⁺ cell phenotype in 8 month old B6.NZBc1c13 bicongenic mice with B6.NZBc1 and B6.NZBc13 congenic strains.*

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<th>B6</th>
<th>B6.NZBc1</th>
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| % B220⁺          | 56.48 ± 9.02 | 58.16 ± 8.28 | 59.78 ± 7.61 | 51.65 ± 10.01 | 29.25 ± 12.49 ***,
|                  |          |          |          |             | (5)  |
| % B7.1⁺          | 18.67 ± 13.40 (9) | 26.88 ± 6.79 (15) | 29.91 ± 6.42 (5) | 29.42 ± 11.45 (22) | 38.57 ± 5.46 * (5) |
| MFI B7.2         | 34.10 ± 24.05 (9) | 46.10 ± 15.62 (15) | 46.30 ± 6.21 (5) | 44.61 ± 17.60 (22) | 49.97 ± 10.77 (5) |
| MFI ICAM-1       | 308.6 ± 174.6 (9) | 449.4 ± 94.50 (15) | 455.3 ± 38.12 (5) | 476.3 ± 165.0 (22) | 437.7 ± 185.5 (5) |
| % CD69⁺          | 6.06 ± 4.41 (9) | 9.01 ± 2.90 ** (15) | 9.70 ± 4.25 (5) | 14.62 ± 7.19 (22) | 5.52 ± 2.19 ** (5) |
| % CD21lowCD23⁻   | 12.32 ± 3.80 | 14.01 ± 5.35 | 14.31 ± 4.83 | 15.43 ± 7.25 | 35.37 ± 9.66 *** |
| % CD21intCD23⁺   | 66.92 ± 10.75 | 68.79 ± 8.75 | 58.83 ± 3.17 | 64.70 ± 14.14 | 33.25 ± 9.23 *** |
| % CD21hiCD23⁺    | 4.78 ± 3.76 | 4.84 ± 3.99 | 4.36 ± 3.19 | 4.76 ± 2.91 | 0.94 ± 1.01 *** |
| % CD21hiCD23⁻    | 6.45 ± 1.71 | 4.00 ± 2.78 | 10.12 ± 4.42 *** | 3.29 ± 1.60 | 4.93 ± 3.67 |

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1 Results are mean ± SD. Significance level for comparison of B6.NZBc1c13 mice with other mouse strains, as determined by Mann-Whitney non-parametric test.

* p < 0.05, ** p < 0.005, *** p < 0.0005.

2 Numbers of 8 month old mice examined in each group unless otherwise indicated in brackets.

3 Numbers shown in bold indicate significant difference from B6 controls.
4.4.2- B6.NZBc1c13 mice demonstrate a dramatic expansion of DC populations

B6.NZBc1 and/or B6.NZBc13 mice have a number of other cellular abnormalities including splenomegaly, expansion of myeloid DC (mDC), and increased T cell activation (165,212). Therefore, the impact of genetic interactions between loci on chromosomes 1 and 13 on these phenotypes was also assessed in bicongenic mice. As shown in Table 4.2, the splenic weight and number of splenocytes was significantly greater in 8 month old bicongenic mice than their monocongenic counterparts. Nevertheless, the splenic weight of bicongenic mice remained, even though not statistically significant, less than that observed for NZB mice, indicating that additional genetic loci may contribute to this phenotype in NZB mice.

While the proportions of CD4+ T cells, CD8+ T cells and CD11b+CD11c- macrophages were similar in bicongenic mice to those observed in one or both of the monocongenic mouse strains, there was a marked increase in the proportion of CD11c+ DC. The proportion of these cells in bicongenic mice was increased approximately 2 fold as compared to monocongenic strains and represented almost a quarter of splenocytes. To further characterize the phenotype of the expanded DC population, splenocytes were stained with anti-CD11c Ab together with anti-B220 and -NK1.1, or anti-CD11b to identify the B220+CD11c+NK1.1- pDC or CD11c+CD11b+ mDC, respectively. As shown in Figure 4.1, expansions of both pDC and mDC compartments contributed to the increased proportion of DC in bicongenic mice. This increase was most pronounced for pDC where there was a ~5 fold increase in bicongenic mice as compared to monocongenic mice. Staining with PDCA-1 confirmed the identity of the B220+CD11c+NK1.1- cells as pDC (data not shown). Consistent with previous reports
(331), increases in the splenic pDC and mDC compartment were not seen in NZB mice, suggesting that additional genetic loci present in NZB mice suppress this phenotype.

Expansion of the pDC population was not seen in the bone marrow of 8 month old bicongenic mice (%B220<sup>+</sup>CD11c<sup>+</sup>NK1.1<sup>−</sup> cells, B6 = 2.85% ± 1.34, n = 13; B6.NZBc1 = 2.72% ± 1.41, n = 18; B6.NZBc13 = 2.79% ± 1.51, n = 4; B6.NZBc1c13 = 2.29% ± 1.44, n = 17; NZB = 2.34% ± 1.09, n = 6; all p > 0.05 as compared to B6 mice). However, moderate expansion of the bone marrow mDC compartment was observed (%CD11c<sup>+</sup>CD11b<sup>+</sup> cells, B6 = 2.20% ± 1.24, n = 13; B6.NZBc1 = 4.01% ± 1.68, n = 18, p = 0.0035; B6.NZBc13 = 2.44% ± 1.35, n = 4, p > 0.05; B6.NZBc1c13 = 5.57% ± 3.29, n = 17, p = 0.0002; NZB = 1.69% ± 0.59, n = 6, p > 0.05, all p values as compared to B6). Differences in the proportions of pDC and mDC in the spleen, and for mDC in the bone marrow, were already seen in 2 month old B6.NZBc1c13 mice but were much less marked (spleen pDC, B6 = 0.79% ± 0.33, n = 9; B6.NZBc1c13 = 1.34% ± 0.47, n = 11; p < 0.05; spleen mDC, B6 = 2.53% ± 0.86, n = 9; B6.NZBc1c13 = 5.33% ± 1.49, n = 11; p < 0.0005; bone marrow pDC, B6 = 2.09% ± 0.27, n = 6; B6.NZBc1c13 = 2.03% ± 0.49, n = 8; p > 0.05; bone marrow mDC, B6 = 2.37% ± 0.29, n = 6; B6.NZBc1c13 = 3.36% ± 1.15, n = 8; p < 0.05).
**TABLE 4.2**

*Table II. Comparison of the splenic phenotype in 8 month old B6.NZBc1c13 bicongenic mice with B6.NZBc1 and B6.NZBc13 congenic strains
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<td>spleen weight (mg)</td>
<td>109.5 ± 33.8</td>
<td><strong>222.4 ± 56.7</strong> ***3</td>
<td>246.4 ± 130.9 <strong>2</strong></td>
<td>376.5 ± 161.1</td>
<td>508.9 ± 310.6</td>
</tr>
<tr>
<td># splenocytes (x 10⁶)</td>
<td>58.94 ± 19.45</td>
<td><strong>100.3 ± 48.32</strong> *</td>
<td>83.28 ± 25.25 <strong>2</strong></td>
<td>131.8 ± 65.49</td>
<td>136.5 ± 84.07 <strong>2</strong></td>
</tr>
<tr>
<td>% CD4⁺</td>
<td>19.68 ± 3.96</td>
<td>20.61 ± 2.76</td>
<td>18.77 ± 3.25</td>
<td>20.70 ± 3.75</td>
<td>23.16 ± 5.00</td>
</tr>
<tr>
<td>% CD8⁺</td>
<td>10.91 ± 5.43</td>
<td><strong>6.67 ± 3.41</strong></td>
<td>8.96 ± 1.13 *</td>
<td>5.77 ± 2.18</td>
<td>6.07 ± 2.61</td>
</tr>
<tr>
<td>% CD11c⁺</td>
<td>10.33 ± 4.47 (14)</td>
<td>14.36 ± 6.53 <strong>2</strong> (21)</td>
<td>12.91 ± 4.69 *</td>
<td><strong>23.22 ± 9.81</strong> (23)</td>
<td>10.66 ± 1.34 ***</td>
</tr>
<tr>
<td>% CD11b⁺CD11c⁻</td>
<td>2.38 ± 1.06 (11)</td>
<td>3.52 ± 1.45 (13)</td>
<td>1.54 ± 0.73 (4)</td>
<td>2.42 ± 1.08 (11)</td>
<td><strong>11.59 ± 8.25</strong> (8)</td>
</tr>
</tbody>
</table>

¹ Results are mean ± SD. Significance level for comparison of B6.NZBc1c13 mice with other mouse strains, as determined by Mann-Whitney non-parametric test,

* p < 0.05, **p < 0.005, ***p < 0.0005.

² Numbers of 8 month old mice examined in each group unless otherwise indicated in brackets.

³ Numbers shown in bold indicate significant difference from B6 controls.
**FIGURE 4.1 Expansion of dendritic cell population in the bicongenic mice.** Freshly isolated splenocytes from 8 month old B6, B6.NZBc1, B6.NZBc13, B6.NZBc1c13 and NZB mice were stained with anti-CD11c in combination with anti-B220 and -NK1.1 or anti -CD11b antibodies to assess the proportion of plasmacytoid and myeloid DC. Shown are the (A) absolute number of splenic CD11c$^+$ DC, proportion of (B) B220$^+$CD11c$^+$NK1.1$^-$ pDC and (C) CD11b$^+$CD11c$^+$ mDC. Each symbol represents the determination from an individual mouse. Horizontal lines indicate the mean for each population examined. The $p$ values for significant differences between the congenic mouse strains are shown above bars, whereas asterisks represent significant differences between various congenic mice and B6 controls, *$p<0.05$, **$p<0.005$, ***$p<0.0005$. (D) Representative dot plots show the gating regions for NK1.1$^-$ gated B220$^+$CD11c$^+$ pDC (top panel) and CD11b$^+$CD11c$^+$ mDC (bottom panel). Numbers inside the box indicate the proportion of each population.
Increased levels of CD4\(^+\) T cell activation were seen in B6.NZBc1 and B6.NZBc13 mice, with increased proportions of recently activated CD69\(^+\) and memory/effector CD44\(^{hi}\)CD62L\(^{lo}\) cells (165,212). In addition, splenic CD4\(^+\) T cells from B6.NZBc1 mice demonstrate increased proliferation and production of IFN-\(\gamma\) in response to stimulation with histones. Although the proportions of CD69\(^+\) and CD44\(^{hi}\)CD62L\(^{lo}\) CD4\(^+\) T cells in bicongenic mice were similar to those seen in one or both monocongenic parental mice (Figure 4.2A & B), bicongenic mice secreted significantly more IFN-\(\gamma\) in response to stimulation with total histone when compared with B6.NZBc1 mice (Figure 4.2D). This reflected increased responses to all of the histone subcomponents (H1, H2A, H2B, H3, and H4) tested (Figure 4.2E) which achieved statistical significance for H2A. No IL-4 was produced following stimulation with any of the histones tested for any of the mouse strains (data not shown). This did not result from a general inability of these mice to produce IL-4 because IL-4 could be readily detected in the supernatants of wells stimulated with anti-CD3, and the levels of these cytokines were comparable in all four strains (data not shown).
FIGURE 4.2 Splenic T cell activation. (A-B) Freshly isolated splenocytes from 8 month old B6, B6.NZBc1, B6.NZBc13, B6.NZBc1c13 and NZB mice were stained with anti-CD4, and -CD69 antibodies or a combination of -CD44 and -CD62L antibodies to assess T cell activation. Shown are the (A) percent CD69+ cells within the CD4+ subset and (B) percent CD44hiCD62Llo memory cells within the CD4+ subset. Each symbol represents the determination for an individual mouse. Horizontal lines indicate the mean for each population examined. The p values for significant differences between the congenic mouse strains are shown above bars, whereas asterisks represent significant differences between the various congenic mice and B6 controls, *p<0.05, **p<0.005, ***p<0.0005. (C-E) Presence of T cells responsive to nucleosome histone components
in B6.NZBc1 and B6.NZBc1c13 mice. Splenic CD4\(^+\) T cells, isolated from 8 to 9 month old B6 (●), B6.NZBc1 (○), B6.NZBc13 (●), B6.NZBc1c13 (◇) mice, were cultured together with B6 T-cell depleted APC pulsed with total histones, H1, H2A, H2B, H3 or H4. (C) Histone-induced proliferation. Cells were cultured for 48 hours and pulsed for the last 16 hr with \[^{3}H\] thymidine. The stimulation index was calculated by dividing the mean cpm in presence of antigen by the mean cpm in the absence of antigen. Mean background proliferation with T cells + APC only (cpm: B6 = 248 ± 83, n = 7; B6.NZBc1 = 503 ± 350, n = 9, p > 0.05; B6.NZBc13 = 501 ± 272, n = 2, p = ND; B6.NZBc1c13 = 1726 ± 1265, n = 10, p = 0.0002, all p-values as compared to B6). (D-E) Histone-induced IFN-\(\gamma\) production. IFN-\(\gamma\) was measured in the supernatant of cell cultures at 48 hours in the presence of (D) total histone or (E) H1, H2A, H2B, H3 and H4. Background production of IFN-\(\gamma\) (T cells + APCs only) was similar in B6, B6.NZBc1, B6.NZBc13 and B6.NZBc1c13 mice. The \(p\) values for significant differences between mouse strains are shown above bars.
4.4.3- Clinical autoimmune disease is not amplified in bicongenic mice despite altered auto-Ab production

Given the emergence of novel cellular phenotypes in bicongenic mice, it was of interest to determine whether these cellular changes were associated with augmented auto-Ab production. Six to 7 month old B6.NZBc1 congenic mice produce significantly higher titres of IgG anti-histone, -chromatin and -ssDNA Ab than age-matched B6 controls, whereas IgM and IgG anti-chromatin Ab are predominantly produced in B6.NZBc13 mice (165,212). As shown in Figure 4.3A, bicongenic mice demonstrated features of both strains, with levels of IgG auto-Ab that approximated those seen in the B6.NZBc1 mouse strain and the increased levels of IgM anti-chromatin Ab seen in the B6.NZBc13 strain. In general, the levels of IgM auto-Ab in bicongenic mice exceeded those seen in the parental monocongenic mouse strains, which achieved statistical significance for anti-chromatin Ab. However, with the exception of anti-chromatin Ab, the levels of IgM auto-Ab remained lower than those seen in NZB mice. Notably, B6.NZBc1c13 mice produced moderate to high titres of IgA anti-chromatin Ab and low titres of IgA anti-ssDNA and –dsDNA Ab, which were markedly increased as compared to those seen in monocongenic mouse strains and the level of IgA anti-chromatin and – ssDNA exceeded those seen in NZB mice. Thus, genetic interactions between NZB chromosome 1 and 13 result in the novel generation of IgA auto-Abs.
FIGURE 4.3 Auto-Ab levels in various congenic mouse strains. Serum samples from 8 month old B6, B6.NZBc1, B6.NZBc13, B6.NZBc1c13 and NZB mice were assayed for the presence of total IgM, IgG and IgA as well as IgM, IgG or IgA anti-chromatin, ssDNA and dsDNA Ab. Each symbol represents the determination from an individual mouse. Horizontal lines indicate the mean for each population examined. The p values for significant differences between the congenic mouse strains are shown above bars, whereas asterisks represent significant differences between the various congenic mice and B6 controls, *p<0.05, **p<0.005, ***p<0.0005.
Despite the development of novel cellular and auto-Ab phenotypes, bicongenic mice demonstrated similar amounts of IgG deposition and types of light microscopy changes in their kidneys to those observed in the parental monocongenic mouse strains (Figure 4.4). Furthermore, although NZB chromosome 1 is reported to contain a genetic locus that facilitates anti-RBC Ab production in crosses with other lupus susceptibility loci (157), anti-RBC Abs were not produced in either B6.NZBc1 or B6.NZBc1c13 mice (%RBC IgM⁺, B6 = 1.39% ± 0.95, n = 4; B6.NZBc1 = 2.10% ± 0.87, n = 7; B6.NZBc1c13 = 2.23% ± 0.92, n = 8; all p > 0.05 as compared to B6: %RBC IgG⁺, B6 = 0.67% ± 0.29, n = 4; B6.NZBc1 = 0.73% ± 0.46, n = 7; B6.NZBc1c13 = 0.77% ± 0.26, n = 8; all p > 0.05 as compared to B6). Taken together, these findings indicate that the immune abnormalities that develop in bicongenic mice do not significantly impact on clinical autoimmunity in these mice.
FIGURE 4.4 Renal involvement in 8 month old B6.NZBc1c13 mice. (A) Immunofluorescence scores of frozen kidney sections stained with anti-IgG. Sections were graded as follows: grade 0, no or only trace deposits; grade 1, mesangial deposits; grade 2, mesangial and segmental capillary wall deposits; grade 3, diffuse mesangial and capillary wall deposits; grade 4, crescents. (B) Glomerular scores of kidneys fixed in formalin, paraffin embedded, sectioned, and stained with PAS. Sections were graded as: grade 0, normal glomeruli; grade 1, mesangial expansion and/or proliferation; grade 2, focal segmental proliferative glomerulonephritis; grade 3, diffuse proliferative glomerulonephritis; and grade 4, diffuse proliferative glomerulonephritis with crescents. Each symbol represents the determination from an individual mouse. Horizontal lines indicate the mean for each population examined.
4.4.4 *In-vivo cytokine production in bicongenic mice*

Elevated levels of BAFF, such as those seen in BAFF transgenic mice, are associated with increased levels of total IgA and IgA ANA (107). We therefore investigated whether bicongenic mice have increased levels of BAFF. Serum BAFF levels correlate poorly with BAFF production in NZB mice, presumably due to consumption of BAFF by various B cell populations (E. Pau *et al.*, manuscript in preparation). Therefore splenic *baff* mRNA levels were assessed by qRT-PCR and BAFF protein expression by immunofluorescence microscopy. There was a ~7.5 fold increase in *baff* mRNA levels in bicongenic as compared to B6 mice, which was comparable to or greater than that seen in NZB mice (*Figure 4.5B*). Consistent with the increased levels of *baff* mRNA, staining of spleen sections revealed a marked increase in the number of BAFF-producing cells in bicongenic mice (*Figure 4.5A*). These cells were predominantly located in the red pulp and were CD11c⁺CD11b⁺, indicating that the expanded population of mDC leads to the increased levels of BAFF in bicongenic mice.

To investigate whether the mDC or pDC population in bicongenic mice had been stimulated to produce other pro-inflammatory cytokines, splenic TNF-α levels were assessed by qRT-PCR and tissue immunofluorescence. Similar to BAFF, there were increased levels of *tnfα* mRNA in the spleens of bicongenic as compared to B6 mice. However, the increase in *tnfα* levels was only ~2 fold and much less than observed for *baff*. Staining of spleen sections for TNF-α revealed an increased number of TNF-α-secreting cells in bicongenic mice. Surprisingly, the majority of these were CD11c⁺B220⁺ pDC and not CD11c⁺CD11b⁺ mDC (*Figure 4.5A* and data not shown).
FIGURE 4.5  Production of excess BAFF and TNF-α, and reduced levels of IFN-α in the spleens of B6.NZBc1c13 mice. (A) To determine the presence of mDC in the spleen, sections were stained with biotinylated anti-CD11b and FITC anti-CD11c. (left panel). Cell populations producing BAFF and TNF-α production were characterized by staining with biotinylated anti-CD11b, FITC anti-CD11c with either rabbit IgG anti-BAFF followed by AMCA-conjugated goat anti-rabbit IgG Ab (middle panel) or goat IgG anti-TNF-α followed by AMCA-conjugated bovine anti-goat IgG Ab (right panel). Biotin staining was revealed using rhodamine-conjugated streptavidin as a secondary reagent. Arrows in the left panels indicate the presence of CD11b⁺CD11c⁺ mDC in the
spleen sections whereas arrows in the right panels indicate the location of BAFF-producing CD11b⁺CD11c⁺ mDC and TNF-α-producing B220⁺CD11c⁺ pDC. Scale bar, 100µm. (B) Increased BAFF and TNF-α mRNA expression in B6.NZBc1c13 splenocytes. (C) Reduced IFN-α mRNA expression in B6.NZBc1c13 splenocytes. Relative mRNA expression of genes of interest normalized to β-actin mRNA expression in freshly isolated splenocytes from 8 month old B6, B6.NZBc1, B6.NZBc1c13 and NZB mice. Each point represents the determination from an individual mouse. The p values for significant differences between B6.NZBc1c13 or NZB to B6 controls, were determined by Mann-Whitney non-parametric test.
Plasmacytoid DC are the predominant producers of IFN-α (388), which has been shown to exacerbate autoimmunity in NZB and NZB/W mice (330,332,389). To determine whether the expansion of pDC in bicongenic mice is associated with increased levels of this cytokine, splenic levels of representative type I IFN and IFN-induced genes were examined by qRT-PCR. As shown in Figure 4.5C, the levels of expression of these genes were normal or reduced in 8 month old bicongenic mice as compared to B6 mice. Thus, the pDC in bicongenic mice do not appear to be generally activated. Consistent with this concept, the levels of CD86 on the CD11c+CD11b- cell population, which is mostly pDC (see Figure 4.1 and data not shown), were reduced as compared to those in B6 mice (MFI B7.2, B6 = 85.40 ± 16.17, n = 12; B6.NZBc1c13 = 70.29 ± 21.45, n = 14; p = 0.0124). Similar findings were observed for the CD11c+CD11b+ mDC population (MFI B7.2, B6 = 169.20 ± 57.29, n = 12; B6.NZBc1c13 = 91.19 ± 34.15, n = 14, p = 0.0008).

4.4.5- IFN-α production appears to be inhibited in older bicongenic mice

In lupus, secretion of IFN-α by pDC is induced by uptake of immune complexes containing DNA or RNA, resulting in TLR engagement (85,390-392). Thus, the relative absence of IFN-α secretion in bicongenic mice could reflect reduced activation of these cells as a consequence of lower levels of these immune complexes or an impaired ability of pDC to be stimulated by these complexes. Since bicongenic mice have increased levels of ANA and deposition of immune complexes in their kidneys, it seemed unlikely that the absence of pDC activation resulted from a lack of immune complexes; therefore, the ability of pDC to respond to TLR stimulation was assessed. To this end, freshly
isolated splenocytes from 8 week and 8 month old mice were incubated with the TLR-9-ligand, ODN 2216, which has been shown to induce IFN-α secretion by pDC, or ODN 1826, which has been shown to induce TNF-α secretion in a variety of cell types, as a control (Figure 4.6). At 8 wks age, TLR-induced IFN-α and TNF-α was similar in bicongenic and B6 mice. Despite the ~5 fold expansion of pDC in 8 month old mice, levels of IFN-α secretion were reduced ~2 fold in bicongenic mice as compared to B6 mice, indicating a marked reduction in secretion of IFN-α on a per cell basis in bicongenic mice. In contrast, similar levels of TNF-α were secreted by TLR-stimulated splenocytes from 8 month old B6 and bicongenic mice. Taken together, these findings suggest that IFN-α production by TLR-stimulated pDC is inhibited in older bicongenic mice.
FIGURE 4.6 IFN-α and TNF-α production following stimulation of splenocytes with TLR ligands. Freshly isolated splenocytes from (A) 8 week or (B) 8 month old B6 or B6.NZBc1c13 mice were stimulated with ODN 1826 control, ODN 1826, ODN 2216 control or ODN 2216 for 48 h. Levels of IFN-α and TNF-α in the culture supernatants were determined using ELISA. Each symbol represents the determination from an individual mouse. The p values for significant differences between B6 and B6.NZBc1c13 mice are shown above bars.
4.4.6- B cells in B6.NZBc1c13 mice are hyper-responsive to TLR signals

DC expansion is seen in mice that are hyper-responsive to various TLR ligands including: TLR-7 transgenic (87), Tir8 gene deleted (88), and Mer$^{kd}$ mice (393). In these mice, one of the features of this hyper-responsiveness is increased B cell proliferation following TLR stimulation with relevant ligands. Therefore, to investigate whether bicongenic mice are hyper-responsive to TLR signals, freshly isolated splenocytes from young 8 week old mice were labeled with CFSE and stimulated with various TLR ligands. B cell proliferation was quantified by flow cytometry, gating upon B220$^+$ cells that had undergone at least one cycle of proliferation. As shown in Figure 4.7, bicongenic B cells demonstrated increased proliferation, to a wide variety of TLR ligands that bound TLR-7 (R837), TLR-3 (Poly I:C), TLR-9 (ODN 2216, ODN1826), and TLR-4 (LPS). Similar results were obtained for splenic proliferation, as measured by [$^3$H]-thymidine incorporation (data not shown). This was not due to differences in the proportion of B cells in the follicular or marginal zone B cell compartment, because the sizes of these populations were similar between B6 and bicongenic mice at 8 wks of age (data not shown).
FIGURE 4.7 B cell hyper-proliferation in response to TLR stimulation. Freshly isolated splenocytes from 8 week old B6 and B6.NZBc1c13 mice were stained with CFSE and stimulated with imiquimod (R837), poly I:C, ODN 1826 control, ODN 1826, ODN 2216 control, or ODN 2216, or LPS for 3 d and their proliferation is measured by CFSE dilution on gated B220+ B cells. Background proliferation with media alone has been subtracted and was slightly increased in B6.NZBc1c13 as compared to B6 mice (mean percentages of B220+ cell divided: B6 = 1.29% ± 0.76, n = 11; B6.NZBc1c13 = 3.54% ± 1.41, n = 11, p = 0.0008 as compared to B6). Each symbol represents the determination from an individual mouse. The p values for significant differences between B6 and B6.NZBc1c13 mice are shown above bars.
4.5- Discussion

The current experiment was undertaken to determine whether genetic loci on NZB chromosomes 1 and 13, which we have previously shown are associated with many of the altered B cell phenotypes in NZB mice, are sufficient to fully recapitulate the NZB B cell phenotype. We show that this is not the case. Expansions of the splenic MZ and B1a B cell populations observed in NZB, and to a lesser extent in B6.NZBc13, mice were attenuated in bicongenic mice. Thus, other genetic loci must contribute to the altered B cell activation and selection in NZB mice. In this connection, it should be noted that in our mapping study only 30-50% of the variance in the B cell activation phenotypes examined was explained by loci on chromosomes 1, 13, and 17 (adjacent to MHC with H2b promoting activation) (126).

Genetic loci on NZB chromosome 1 appeared to have a dominant effect on MZ and B1a B cell selection. This could reflect the presence of genetic polymorphisms that alter B cell signaling or enhance provision of T cell help in the NZB chromosome 1 interval. B6.NZBc1 mice share the same SLAM locus as B6.Sle1b mice, which have a genetic polymorphism in their Ly108 allele that leads to decreased Ca^{2+} mobilization following Ig receptor engagement (168,170). This could impact B cell selection in B6.NZBc1 mice, resulting in decreased recruitment of cells into the B1a and MZ B cell subsets. Alternatively, B cell hyper-responsiveness to TLR signals in B6.NZBc1c13 mice could promote movement of B cells out of these compartments. In mice with the Y autoimmune accelerator allele or a TLR-7 transgene, similar B cell hyper-responsiveness to TLR-7 stimulation is associated with contraction of the MZ (87,143). Finally,
differences in the quantity or quality of T cell help could facilitate recruitment of cells out of these compartments. This is supported by our observations in NZB mice, where introduction a CD40L gene deletion leads to an expanded MZ population (E. Pau et al., manuscript in preparation).

Surprisingly, bicongenic mice develop several novel phenotypes that are either absent in the monocongenic strains or markedly exacerbated in bicongenic mice. These include expansion of DC subsets, increased IgA auto-Ab production, and elevated BAFF. In bicongenic mice, dramatic increases in the size of the mDC and pDC populations were observed. While both monocongenic mouse strains demonstrated mild increases in the mDC compartment at 8 month, changes in the pDC compartment were only seen in bicongenic mice. Notably, the levels of DC in bicongenic mice were comparable to those observed in mice with various genetic manipulations that lead to increased TLR activation such as TLR-7 transgenic (87), Tir8 knockout (88), and MerKd mice (393). In these mouse strains B cell hyper-proliferation to various TLR ligands has been reported (87,88), which is similar to that seen for B6.NZBc1c13 B cells. This observation raises the possibility that TLR hyper-responsiveness drives expansion of the DC populations in B6.NZBc1c13 mice, either by directly inducing activation or migration of DC into the spleen (394,395), or indirectly by increasing pro-inflammatory factors such as IFN-γ, that have been shown to recruit and/or promote survival of DC (396-398). Our preliminary results indicate that this hyper-responsiveness maps to an interval on NZB chromosome 1 extending from 96 to 100 cM from the centromere (Y. Cheung et al., unpublished observations). Interestingly, this interval contains the candidate gene,Ifi202, a transcriptional regulator that has been shown to lead to increased LPS-induced NF-κB
activation in dendritic cells (399), which could have a more general impact on TLR responses. If relevant, NZB chromosome 1-mediated TLR hyper-responsiveness appears to be insufficient to lead to the marked expansion of DC characteristic of bicongenic mice, requiring genetic contributions from NZB chromosome 13 for the full expression of this phenotype. We have not yet examined TLR responses in B6.NZBc13 mice however our preliminary data suggests that there is impaired clearance of apoptotic debris in these mice (E. Pau et al, manuscript in preparation). Thus, immune defects leading to increased amounts of, and/or enhanced responses to, apoptotic debris may act synergistically to produce the DC phenotype in bicongenic mice. Alternatively, increased expression of endogenous retroviruses associated with a genetic locus on NZB chromosome 13, Sgp3, could lead to increased levels of viral RNA that promote the DC phenotype through stimulation of TLR-7 (218).

BAFF levels were markedly increased in bicongenic mice as compared to B6 mice and were at least as high as those seen in NZB mice. In BAFF transgenic mice there are increased levels of total serum IgA and IgA auto-Ab, and BAFF has been shown to facilitate class switching to IgA production (107,400). Thus, it is likely that the increased IgA levels in NZB and bicongenic mice arise, at least in part, from increased BAFF. At present it is unclear what is driving this BAFF production in bicongenic mice. Although IFN-α has been shown to enhance BAFF production (401), the low levels of IFN-α and IFN-induced gene expression in bicongenic mice suggest that IFN-α does not play a significant role in BAFF induction. Uptake of circulating Ag, such as apoptotic debris, has also been shown to promote localization of mDC-like cells to the spleen (402) and induce their BAFF expression (392,402). These cells have been shown to localize
initially to the MZ, where they cluster with B cells inducing their Ab secretion. In bicongenic mice, BAFF-producing cells are scattered throughout the red pulp and are not found in clusters with Ab-producing B cells, however it is possible that their localization in the MZ was a transient state or were already localized in the spleen and became activated \textit{in-situ}.

Despite the marked expansion of the pDC subset in bicongenic mice, levels of type I IFN did not appear to be increased. This may be relevant to the lack of severe glomerulonephritis in bicongenic mice, since even modest increases in IFN-\(\alpha\) have been shown to markedly accelerate kidney disease (330). There are several potential explanations for the lack of IFN-\(\alpha\) production in older bicongenic mice: 1) Chronic activation of pDC may lead to their desensitization to TLR-signaling, resulting in impaired production of IFN-\(\alpha\) in older mice (403). However, the absence of comparable inhibition for TNF-\(\alpha\) production in older mice argues against this possibility. 2) Altered signaling in the pDC of bicongenic mice could lead to impaired generation of IFN-\(\alpha\), while preserving TNF-\(\alpha\) secretion. Both B6.NZBc1 and B6.NZBc13 mice have intrinsic B cell functional abnormalities consistent with genetic polymorphisms that impact on B receptor signaling ((233) and C. Loh \textit{et al.} manuscript in preparation). pDC, unlike mDC, have a B cell receptor-like signaling pathway down-stream of their Fc\(\gamma\)R and activation of this pathway has been shown to inhibit TLR-mediated secretion of IFN-\(\alpha\) (325). Thus, genetic polymorphisms that lead to altered signaling in B cells could impair IFN-\(\alpha\) secretion by pDC. 3) Secretion of IFN-\(\alpha\) by pDC could be suppressed by other cytokines. Both TNF-\(\alpha\) and IL-10 have been shown to inhibit IFN-\(\alpha\) secretion by pDC (404,405). This possibility is compatible with the observation that secretion of IFN-\(\alpha\) by
bicongenic splenocytes is reduced at 8 month of age, when increased levels of TNF-α mRNA are detected.

Regardless of the mechanisms leading to the impaired IFN-α secretion in bicongenic mice, the data reported herein indicate that expansion of pDC and the presence of anti-DNA Ab need not be associated with increases in IFN-α production. These findings suggest that there is additional complexity in the generation and regulation of IFN-α, and characterization of these processes may provide important insights into the immune dysregulation that promotes SLE.
Acknowledgements: We thank Dr. Eleanor Fish for generously providing the PDCA-1 Ab.
CHAPTER 5: General Discussion and Future Directions

My investigations sought to identify the lupus susceptibility loci associated with the NZB chromosome 1 interval that are located between 35cM to 106cM (61.9 – 190.5 Mb) and determine how they interact with each other and loci on NZB chromosome 13 to produce the lupus phenotype. Prior to my experiments, Dr. Wither’s laboratory had shown that there were at least two lupus susceptibility loci on NZB chromosome 1, one located between 35-82cM and another one located between 82-106cM that led to production of anti-nuclear antibodies and renal disease in B6.NZBc1(35-106) congenic mice (165).

In Chapter 2, I investigated which cell populations had altered function as a result of these allelic polymorphisms, by creating mixed hematopoietic chimeric mice. My results demonstrated that there were intrinsic functional defects in both T and B cells in B6.NZBc1(35-106) mice. I showed that an intrinsic B cell defect was required for B cells to be efficiently recruited in germinal centres and differentiate into autoantibody producing plasma cells, despite the presence of auto-reactive T cells in mixed chimeric mice. This experiment demonstrated the importance of B cell defects in the generation of the lupus phenotype, a finding that has been recently substantiated in human lupus with the identification of single nucleotide polymorphisms in multiple genes that are predominantly expressed in B cells with an increased risk for SLE. The nature of the B cell abnormalities in these mice led me to postulate that B cell anergy induction was defective in B6.NZBc1(35-106) mice. In subsequent experiments, using the anti-HEL Ig Tg x sHEL system, this postulate has been confirmed and the genetic locus associated with this breach of tolerance localized to the 96-100 cM interval (N. Chang et al,
unpublished observation). It is presently unclear whether additional genetic loci outside this interval also contribute to the breach of B cell tolerance in B6.NZBc1(35-106) mice. My mixed chimeric experimental data also suggested that B6.NZBc1(35-106) mice have intrinsic T cell functional defects that lead to increased spontaneous T cell activation and priming if IFN\(\gamma\)-producing histone-reactive T cells in-vivo.

Experiments outlined in Chapter 3 sought to further investigate the location of the genetic polymorphisms that were associated with production of auto-antibodies and renal disease in B6.NZBc1(35-106) mice and assessed the impact of these polymorphisms on T cell activation in-vivo and T cell function in-vitro. Using a series of subcongenic mice, I found surprising genetic complexity on NZB chromosome 1. Instead of just the two lupus susceptibility loci previously identified and proposed in the NZB 35-106 cM chromosome 1 interval, I found that there are at least four lupus susceptibility loci in this interval that are located in the 70-82cM (Locus 1), 88-96cM (Locus 3), 96-100cM (Locus 2) and 102-106cM (Locus 4) regions. In addition, the autoimmune phenotype of B6.NZBc1(35-106) mice is modulated by the presence of a suppressor locus located between 35-62cM. These results are summarized in Figure 5.1.
FIGURE 5.1 Genetic maps of chromosome 1 congenic lines used in our studies. B6.NZBc1(43-85), B6.NZBc1(96-100), B6.NZBc1(88-100), B6.NZBc1(70-100), B6.NZBc1(35-102) and B6.NZBc1(35-106) mouse strains are shown. Thick and thin lines denote NZB and B6 regions, respectively. Dashed lines indicate regions of undefined origin. Polymorphic microsatellite markers and single nucleotide polymorphism (SNP) markers were used to discriminate between NZB and B6 DNA at the termini of the regions according to the NCBI m37 mouse genome assembly (<www.ensembl.org>). Markers are shown to the right of each chromosome representation, with the position of these markers shown to the left. A scale is shown on the far left of the figure together with the position of four lupus susceptibility locus and two lupus suppressor loci identified in our previous experiments. Each of the six loci is highlighted in grey boxes to facilitate comparison with our congenic mouse lines. Genes shown on the left of each grey box are the possible candidate genes located in locus 2.
Our current results indicate the lupus susceptibility loci in the 70-82cM (Locus 1) and 96-100cM (Locus 2) interval alter T cell function. Locus 1 by itself appears to be insufficient to produce ANAs but leads to increased T cell activation and an increased number of germinal centres. Consistent with altered T cell function in this locus, B6.NZBc1(43-85) mice demonstrated a decreased threshold for IFN-\(\gamma\) production following stimulation with anti-CD3, and T cell proliferation and IFN-\(\gamma\) production was augmented in B6.NZBc1(70-100) mice as compared to B6.NZBc1(96-100) mice. Within the 70-82cM interval, there are 268 known and predicted genes among which \textit{Roquin} (\textit{Rc3h1}), \textit{Cxcr4}, \textit{Ctse} and \textit{Ptprv} represent attractive candidate genes. \textit{Roquin} is particularly attractive candidate, since \textit{sanroque} mice carrying a M199R mutation in the Roquin protein exhibit a lupus like phenotype including the generation of ANAs and glomerulonephritis (101). Notably, these mice have increased numbers of germinal centres and T\textsubscript{FH} cells, which are found within the compartment that is expanded in B6.NZBc1(43-85) and B6.NZBc1(70-100) mice. In further support of a role for T\textsubscript{FH} in our congenic mouse strains, recent experiments in the Wither laboratory have found that the proportion of T\textsubscript{FH} and IL-21 production is increased in B6.NZBc1(70-100) (N. Talaei \textit{et al.}, unpublished observation). Ongoing experiments will determine whether Roquin expression is altered in B6.NZBc1(43-85) and B6.NZBc1(70-100) T cells and/or whether there are polymorphisms in the \textit{Roquin} locus of NZB as compared to B6 mice. Similar experiments could be performed to assess expression of other candidate genes within the 70-82 interval. However, it is possible that none of the candidate genes outlined above represent the relevant susceptibility locus. Consequently, as an alternative approach gene expression arrays on purified T cells could also be performed to examine the genes that
are differentially expressed in the congenic mice carrying this interval, followed by qRT-PCR that specifically examine potential candidate genes that exhibited differential expression in the arrays.

Available evidence suggest that the most likely candidate gene within Locus 2 (in the 96-100 cM interval) is a slam polymorphism, most likely in Ly108, as proposed by another group examining lupus candidate genes in the lupus prone NZM2410 mouse model using B6.Sle1 congenic mice (168). As outlined in Chapter 3, my data together with that published in the literature indicates that the two additional candidate genes in the 96-100 cM interval do not appear to contribute significantly to the autoimmune phenotype in B6.NZBc1(96-100) mice. Although generation of further subcongenic mice derived from the B6.NZBc1(96-100) mouse strain would enable this to be concluded more definitively, to date I have been unable to generate mice with crossovers in this interval. As an alternative approach generation of B6 mice with the autoimmune Ly108 allele knocked in could be used to determine whether this gene alone is sufficient to reconstitute the lupus phenotype. Another approach that could be used would be to retrovirally infect bone marrow to induce increased expression of the Ly108.1 splice variant in B6 mice that is thought to produce the lupus phenotype or knock down expression of the Ly108.1 splice variant in B6.NZBc1(96-100) mice.

My results also suggest the presence of two additional susceptibility loci on NZB chromosome 1, one located between 88-96cM (Locus 3) and one in the 102-106cM (Locus 4). In Locus 3, there are 23 known genes of which the gene SH2 domain-containing protein 1B (Sh2d1b), encoding the EAT-2A adapter protein that binds to the cytoplasmic tails of SLAM, CD224, CD84 and Ly108, represents the most attractive
candidate (406). EAT-2A is mainly expressed in NK cells (but also in B cells, mast cells and macrophages) and has been shown to inhibit NK cell function through tyrosine phosphorylation (407,408). In fact, Sh2d1b deficient mice (EAT-2A-/−) exhibited enhanced NK cell cytotoxicity as well as increased IFN-γ secretion upon stimulation with anti-CD16, NKG2D, Ly49D and CD224 (408). Given the limited number of genes in this interval, it is quite feasible to directly assess the expression levels of any possible candidate genes in various lymphocyte populations using RT-PCR (contrasting B6, B6.NZBc1(96-100) and B6.NZBc1(88-100) mice) and to sequence RNA transcripts seeking coding polymorphisms. Of note, Sh2d1b is highly polymorphic with at least 76 known SNPs in various mouse strains, increasing the likelihood that polymorphisms between B6 and NZB mice exist. It will also be of interest to further assess the numbers and function of NK cells in these mice.

There are also a limited number of potential candidate genes in the 102-106 cM interval that contains locus 4. Indeed, there are only 12 known and predicted genes located in this interval, with transforming growth factor beta 2 (Tgfb2) being the one with best defined immunologic function. TGFβ2 has been shown to play an important role in the generation of APCs that induce various regulatory T cell subsets and in the expansion of CD4+CD25+FoxP3+ cells and TGFβ1 secreting regulatory T cell subsets (409). Thus it is possible that this locus affects the generation of one or more of these cell populations leading to enhanced autoimmunity. This possibility is compatible with the observation that this locus leads to enhanced the production of IgG anti-nuclear antibodies as well as the formation of germinal centres but does not appear to alter proliferation and/or IFN-γ production by anti-CD3 stimulated T cells in-vitro.
Comparison of the phenotypes of B6.NZBc1(35-106) and B6.NZBc1(35-102) with B6.NZBc1(70-100) and B6.NZBc1(62-102) congenic mice, indicates the presence of a suppressor locus in the 35-62cM interval that significantly attenuates cellular activation, germinal centre number and size, development of renal disease, and IgG autoantibody production. In addition, T cell proliferation and IFN-γ production following anti-CD3 stimulation in-vitro was reduced by this locus. These findings suggest that this locus affects T cell function. Currently this interval contains a large number of genes making identification of the relevant genetic polymorphism difficult. Therefore ongoing experiments are seeking to narrow this interval through generation of additional subcongenic mouse strains.

Figure 5.2 provides a tentative scheme for how the different genetic loci on NZB chromosome 1 interact with each other to produce the autoimmune phenotypes in the various sub-congenic mouse strains. In this scheme locus 2 plays a critical role in the breach of tolerance to nuclear antigens through effects on B cell anergy induction and T cell function leading to priming of the histone-reactive T cells that provide support of IgG anti-nuclear antibody production. Locus 1 and 3 appear to augment T cell help for autoantibody production and renal disease by leading to enhanced generation of T_{FH} cells and production of additional pro-inflammatory cytokines such as IL-21 and IL-17. The suppressor locus appears to act by inhibiting the generation of activated T cell populations and pro-inflammatory cytokines in-vivo, but is insufficient to completely correct the B cell tolerance defect in these mice since mice with the locus together with susceptibility Loci 1-3 continue to make IgM autoantibodies. Finally, Locus 4 acts to
restore T cell activation in-vivo, possibly by affecting the generation of regulatory T cell subsets.
FIGURE 5.2 Proposed mechanisms for the interactions between the four lupus susceptibility loci and two suppressor loci on NZB chromosome 1. Results show that the two major loci, Locus 1 and 2, promote the development of lupus by disrupting T cell function where as Locus 3 enhance the formation of germinal centres and production of IgG ANAs and Locus 4 acts to increase the generation of pathogenic autoantibodies.
Experiments outlined in Chapter 4 sought to determine if the loci from NZB chromosome 1, together with those from NZB chromosome 13 were sufficient to restore the abnormal B cell phenotypes observed in NZB mice. This research question was based on the results of various mapping studies that showed that loci from both chromosome 1 and 13 were linked to various aberrant B cell phenotypes and dysregulated autoantibody production in NZB mice (125,126,153). Surprisingly, despite the presence of multiple susceptibility loci from both chromosomes in bicongenic mice, the levels of B cell activation and autoantibody production as well as the severity of lupus nephritis were comparable to the single congenic parents. This finding suggests that other loci in NZB mice must contribute to the altered B cell phenotype and is compatible with the observation that genetic loci on chromosome 1 and 13 were insufficient to explain all of the variance in these phenotypes in the mapping study.

Nevertheless, bicongenic mice also exhibited some unexpected phenotypes including marked expansion of splenic pDC and mDC populations, elevated levels of BAFF and IgA autoantibody production. As these phenotypes together with a reduced proportion of marginal zone B cells are seen in mice with enhanced responses to TLR engagement (410), I investigated whether there was evidence for TLR hyper-responsiveness in bicongenic mice. Consistent with this possibility, bicongenic B cells demonstrated enhanced proliferative responses to TLR3, TLR7, and TLR9 ligands. Although this findings suggested that the DC in bicongenic mice may be similarly hyper-responsive, my initial studies did not address this possibility. However, since this time, in collaboration with Evelyn Pau another member of the Wither laboratory, I have extended the findings to DCs. For these experiments DCs were expanded from bone
marrow by culture with FLT3L for 7 days and then stimulated with various TLR ligands. TLR responses were assessed by measurement of cytokine production 1 day later. In bicongenic mice increased production of TNF\(\alpha\) and/or IFN\(\alpha\) was observed following stimulation with TLR3, TLR7, and TLR9 ligands. Additional experiments performed by other members of the laboratory have demonstrated that the TLR3 hyper-responsiveness arises from the B6.NZBc13 parent whereas the TLR7 and TLR9 hyper-responsiveness arises from the B6.NZBc1 parent (C. Loh et al, unpublished observation).

Despite the expansion of pDC and mDC in bicongenic and the proposed role of these cells in secretion of the various pro-inflammatory cytokines that are thought to enhance the lupus phenotype, bicongenic mice had relatively mild renal disease and demonstrated no increase in mortality up to 12 month of age. These findings contrast with those of other mice that are hyper-responsive to TLR ligands and suggests that some form of regulation of pro-inflammatory cytokine production is occurring (217,411). Consistent with this possibility reduced production of IFN\(\alpha\) in 8 month old mice was observed both in-vivo and in-vitro following TLR stimulation. Findings outlined above and in Chapter 4, indicate that this does not result from an intrinsic inability of DCs from bicongenic mice to produce INF\(\alpha\) with appropriate stimulation. Nor does it appear to arise from DC exhaustion, because DCs from 8 month old mice have been stimulated in-vivo to produce BAFF and TNF\(\alpha\), indicating that they are not refractory to activation. Therefore, the most likely possibility is that IFN\(\alpha\) production is regulated in bicongenic mice. Notably, TNF\(\alpha\) and IL-10 have been shown to inhibit IFN\(\alpha\) production by pDC in-vitro (405,412). Thus, it is possible that the increased production of TNF\(\alpha\) in bicongenic mice inhibits IFN\(\alpha\) production by pDC.
The proposed scheme for the interaction between chromosome 1 and 13 genetic loci to produce the phenotypic abnormalities in bicongenic mice is shown in Figure 5.3.
FIGURE 5.3 Epistatic interactions between lupus susceptibility loci on NZB chromosome 1 and 13 results in the expansion of plasmacytoid and myeloid dendritic cells with altered autoimmune phenotype.
Subsequent experiments that could be performed to address various aspects of this model include: 1. Administration of an adenoviral vector that produces IFNα to examine whether the attenuated disease phenotype in bicongenic mice is due to the failure to produce IFNα. 2. Addition of anti-TNFα and/or IL-10 Ab to splenocyte cultures from young and old bicongenic mice that are stimulated with various TLR ligands to determine whether this restores production of IFNα. 3. Addition of splenocytes from young and old mice to TLR stimulated DC to determine whether they inhibit IFNα production, followed by identification of the relevant cytokines and/or cell populations if inhibition is seen.

In human lupus there are patients that have a similar phenotype to B6.NZBc1c13 mice, with elevated levels of IgG anti-nuclear autoantibodies but lacking clinical manifestation of the disease (413). It would be very interesting to determine whether IFNα production and/or other pro-inflammatory cytokine production is altered in these patients and if so whether this is due to peripheral blood mononuclear cells populations that actively regulate IFNα production.
CHAPTER 6: References


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peritoneal B-1a cells and lupus nephritis map to different loci. *J Immunol.* 175:936.


