Identification of genetic polymorphisms that promote autoimmunity on New Zealand black (NZB) chromosome 1 and their mechanisms of action

by

Nafiseh Talaei

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

Department of Immunology

University of Toronto

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2016

ABSTRACT

Systemic Lupus Erythematosus (SLE) is a multisystem autoimmune disease characterized by the production of autoantibodies and the development of an immune complex-mediated glomerulonephritis (GN). The New Zealand Black (NZB) mouse is considered to be an excellent model of SLE. Identification of susceptibility loci and the mechanisms through which they act to produce autoimmunity has been greatly aided by the study of congenic mouse strains, in which homozygous intervals containing these loci have been introduced onto a non-autoimmune B6 genetic background. Genetic loci on NZB chromosome (c) 1 lead to antinuclear antibody (ANA) production and glomerulonephritis (GN). Previously our laboratory showed that mice with a homozygous NZB 70 to 100 cM c1 interval introgressed onto the B6 background develop fatal GN. Using a series of congenic mice with shorter NZB c1 intervals, the laboratory
has demonstrated that there are at least three lupus susceptibility loci located within this region that are sufficient to lead to high titer anti-nuclear auto-antibody production and severe life-threatening GN. In this thesis I have sought to further characterize the cellular abnormalities and identify the genetic polymorphisms that produce the phenotype in c1(70-100)mice. In the second chapter, I have characterized the immune functional defects associated with the severity of disease in a series of sub-congenic mice with shorter intervals derived from this mouse strain. I showed that the severity of renal disease parallels expansion of Th1, Th17, and Tfh pro-inflammatory cell subsets. I also demonstrate that expansion of these cell subsets results from altered T cell and dendritic cell functional abnormalities, which is a consequence of interactions between at least 3 independent genetic loci. Next, I sought to identify the genetic polymorphism that leads to altered dendritic cell function in NZB chromosome 1 congeneric mice. In chapter 3, I provide evidence suggesting that a polymorphism in EAT-2 leads to reduced levels of expression of this molecule in NZB c1 congeneric mice, resulting in increased levels of cytokine secretion by myeloid DC that support expansion of pathogenic T cell subsets.
Acknowledgments

I have several persons I want to thank for their time, patients, guidance, support and encouragement during my PhD program at the University of Toronto. First, I would like to express my sincere appreciation to my supervisor, Dr. Joan Wither, for giving me an opportunity to carry out my graduate studies in her laboratory and for providing me with invaluable source of support, guidance and independence to pursue my research.

I would like to thank members of my supervisory committee, Dr. Eleanor Fish and Dr. Phillipe Poussier, for their feedbacks, suggestions, supports and encouragements. I would like to thank all past and present members of Dr Wither’s lab: Dr. Nan Chang, Dr. Carolina Landolt, Dr. Christina Loh, Dr. Evelyn Pau, Babak Noamani, Timothy Li, Yuriy Baglaenko, Kimberley Lifeso, Gillian Minty, and Kieran Manion for their generous help, support and suggestions, and most of all, friendship. In particular, I want to gratefully acknowledge the help of Dr Nan Chang, who offered much helpful advice and technical assistance. Also, I would like to extend a special thanks to my dear friend, Ramtin, who have always stood by me during this long journey.

Finally, I’d like to thank all my family members. Words cannot even describe how blessed and fortunate I am to have such a supportive and caring family. I want to extend my greatest gratitude to my husband, Reza, and my beautiful daughter, Annisa, for their tremendous support and love in all aspect and tolerance throughout my PhD program. I want to thank my mom for instilling in me such a strong work ethic. Finally, in loving memory of my dear father, Mehdi Talaei, thank you for giving me life and being a great inspiration to me I am proud to be your daughter I miss you so much.
TABLE OF CONTENTS

ABSTRACT ................................................................. ii

TABLE OF CONTENTS ...................................................... v

LIST OF TABLES ........................................................... xi

LIST OF FIGURES .......................................................... xii

LIST OF ABBREVIATIONS ................................................... xv
CHAPTER 1: INTRODUCTION

1.1- Systemic lupus erythematosus

1.1.1- Genetic factors in SLE

1.2 Lupus prone mouse models

1.2.1- MRL/lpr mice

1.2.1.1- Genetic associations/mechanisms associated with the development of lupus in MLR/lpr mouse strain

1.2.2- BXSB mice

1.2.2.1- Genetic associations/mechanisms associated with the development of lupus in BXSB mouse strain

1.2.3- NZ mice

1.2.3.1- Genetic associations/mechanisms associated with the development of lupus in NZ mouse strain

1.2.4- Chromosome 1 congenic mouse strains

1.2.4.1- NZM-derived chromosome 1 congenic mouse strains

1.2.4.2- NZB-derived chromosome 1 congenic mouse strains

1.3- SLAM/CD2 family of receptors

1.3.1- General characteristics
1.3.2- SLAM family polymorphisms and lupus..............................28
1.3.3- SLAM- associated protein (SAP) family adaptors.....................30
1.3.4- EAT-2 ..............................................................................31
1.4- Dendritic cells........................................................................32
1.4.1- Myeloid and plasmocytoid dendritic cell subsets ......................33
1.4.1.2- Role of mDC in the pathogenesis of lupus ............................33
1.4.1.2- Role of pDC in the pathogenesis of lupus.............................35
1.5- Role of T cells in the pathogenesis of SLE..................................39
1.5.1- Role of Th1 cells in the pathogenesis of lupus ............................39
1.5.2- Role of Th17 cells in the pathogenesis of lupus .........................41
1.5.3- Role of Th17 cells in the pathogenesis of lupus ..........................42
1.6- Thesis objectives........................................................................45

CHAPTER 2: T cell and Dendritic cell Abnormalities Synergize to Expand
Pro-inflammatory T cell Subsets Leading to Fatal Autoimmunity in
B6.NZBc1 Lupus-Prone Mice.............................................................48-89

2.1- Abstract....................................................................................49
2.2- Introduction...............................................................................50
2.3- Materials and Methods…………………………………………………………………………52

2.3.1- Ethics statement……………………………………………………………………………52

2.3.2- Mice…………………………………………………………………………………………52

2.3.3- Flow cytometry……………………………………………………………………………52

2.3.4- Detection of cytokine-secreting T cells………………………………………………53

2.3.5- Naïve CD4+ T cell isolation and differentiation………………………………………54

2.3.6- In-vivo differentiation of OVA-specific T cells………………………………………55

2.3.7- Immunofluorescence staining of tissue sections………………………………………55

2.3.8- BMDC isolation and stimulation…………………………………………………………56

2.3.9- In-vitro culture of BMDCs and OVA-specific T cells……………………………56

2.3.10- In-vitro culture of splenocytes and OVA-specific T cells………………………57

2.3.11- Statistical analysis………………………………………………………………………57

2.4- Results……………………………………………………………………………………………58

2.4.1- Expansion of pro-inflammatory CD4+ T cell subsets in NZB c1 congenic Mice…………………………………………………………………………………58

2.4.2- Intrinsic skewing of the immune system towards increased generation of Tfh, Th17 and Th1 cell subsets in c1 congenic mice……………………………69

2.4.3- Altered T cell differentiation in c1 congenic mice results from defects affecting T and non-T cell function…………………………………………………71
2.4.4- DC from c1(88-100) and c1(70-100) mice demonstrate altered function that promotes differentiation of pro-inflammatory T cell subsets.................76

2.5- Discussion........................................................................................................85

CHAPTER 3: Identification of the SLAM Adapter Molecule EAT-2 as a Lupus Susceptibility Gene that Acts through Impaired Negative Regulation of Dendritic Cell Signaling.........................................................91-121

3.1- Abstract...........................................................................................................92

3.2-Introduction......................................................................................................93

3.3- Materials and Methods..................................................................................95

3.3.1- Ethics statement.........................................................................................95

3.3.2- Mice.............................................................................................................95

3.3.3- Flow cytometry...........................................................................................95

3.3.4- BMDC isolation and expansion.................................................................96

3.3.5- Measurement of EAT-2 mRNA expression in BMDC...............................96

3.3.6- Promoter sequencing and Luciferase Assay..............................................97

3.3.7- Transfection of BMDC with EAT-2 siRNAs.............................................98

3.3.8- In-vitro culture of transfected BMDC and OVA-specific T cells..............98

3.3.9-Western Blots..............................................................................................99
3.3.10- BMDC CD40 stimulation ........................................................................99
3.3.11- Phospho-flow .......................................................................................100
3.3.12- Immunoprecipitation ..........................................................................100
3.3.13- Statistical analysis ................................................................................101
3.4- Results ..........................................................................................................102
  3.4.1- A genetic polymorphism in the promoter region of EAT-2 in NZB c1
  congenic mice results in altered expression of EAT .......................................102
  3.4.2- Knockdown of EAT-2 in BMDC from B6 and c1(96-100) mice
  recapitulates the c1(70-100) phenotype .......................................................103
  2.4.3- Reduced levels of EAT-2 in c1 congenic DC result in enhanced IL-12
  production in response to CD40 signaling ..................................................110
  3.5- Discussion .................................................................................................117

CHAPTER 4: General Discussion and Future Directions .........................123-133

References ........................................................................................................134-154
LIST OF TABLES

CHAPTER 1

Table 1.1. Proposed mechanisms and candidate genes implicated to promote SLE………………………………………………………………………………………..3

Table 1.2. Expression pattern, function, and signal transduction effector molecules for the SLAM receptors……………………………………………………………………………….25
LIST OF FIGURES

CHAPTER 1

Figure 1.1- Genetic map of the c1 congenic mouse strains........................................18

Figure 1.2- Comparison of validated lupus susceptibility loci on mouse chromosome 1 in different mouse strains..............................................................23

Figure 1.3- Structural representation of the six core SLAM family members..............27

Figure 1.4- Proposed model of the Th1, Tfh and Th17 cell contribution to SLE pathogenesis.................................................................44

CHAPTER 2

Figure 2.1- Genetic map of the c1 congenic mouse strains studied.........................59

Figure 2.2- c1 congenic mice have an increased proportion of GC B and Tfh cells.....60

Figure 2.3- Expansion of Tfh, Th17 and Th1 cell subsets in c1 congenic mice.........65

Figure 2.4- c1 congenic mice exhibit increased production of cytokines secreted by Tfh, Th1 and Th17 populations.................................................................66

Figure 2.5- Identification of cytokine-producing T cell subsets in c1 congenic mice ....67
Figure 2.6- Enhanced differentiation of pro-inflammatory T cell subsets in c1 congenic mice following OVA immunization…………………………………………………………70

Figure 2.7- Increased differentiation of naïve CD4+ T cells from c1 congenic mice to Th17 and Th1 cells in-vitro ……………………………………………………………………..72

Figure 2.8- Intrinsic T cell functional defects together with altered environmental cues promote the enhanced differentiation of OVA-specific T cell subsets in congenic mice …………………………………………………………………………………….75

Figure 2.9- Splenic mDC from c1(70-100) congenic showed increased production of IL-6 and IL-12, and induce enhanced T cell differentiation in-vitro…………………………..77

Figure 2.10- Myeloid DC from c1(88-100) and c1(70-100) mice demonstrate altered function and an enhanced ability to induce differentiation of Th1 cells. ……………..81

Figure 2.11- Altered production of IL-6 and/or IL-12 by myeloid DC from c1(88-100) and c1(70-100) mice following stimulation with TLR ligands …………………..83

CHAPTER 3

Figure 3.1- A NZB EAT-2 polymorphism leads to decreased expression of EAT-2 in BMDC…………………………………………………………………………………………..105

Figure 3.2- Knockdown of EAT-2 leads to increased production of IL-12 by DC and increased differentiation of OT-II T cells to Th1 cells in-vitro ……………………..107

Figure 3.3- BMDC transfection efficiency as determined by siGLO green indicator…109
Figure 3.4 - Increased production of IL-12 by anti-CD40-stimulated BMDC from c1(88-100) and c1(70-100) mice is recapitulated by EAT-2 knockdown in control cells……112

Figure 3.5 BMDCs express the same level of CD40 in all mouse strains………………115

Figure 3.6- SLAM-mediated inhibition of signaling downstream of CD40 is deficient in BMDC from c1(70-100) mice…………………………………………………………………………………………116

CHAPTER 4

Figure 4.1- Expression levels of the different Ly108 isoforms in c1 congenic mice………………………………………………………………………………………………………125

Figure 4.2- Knock down of Ly108 leads to reduced Th1 and Th17 differentiation in both c1 congenic and B6 mouse T cells…………………………………………………………………………127

Figure 4.3- T cells from c1(70-100) mice show lower expression levels of Rxr-γ after 24h stimulation with anti-CD3 and -CD28…………………………………………………………130
LIST OF ABBREVIATIONS

Ab – Antibody
Ag – Antigen
ANA – Anti-nuclear antibody
APC – Antigen presenting cell
B – B lymphocyte
B6 – C57BL/6
BCR – B cell receptor
B_{reg} – Regulatory B cell
BM – Bone marrow
BMDC – Bone marrow dendritic cells
c – Chromosome
CD – Cluster of differentiation
cM – Centimorgan
DC – Dendritic cell
dsDNA – Double stranded DNA
dTg – Double transgenic
GN – Glomerulonephritis
GM-CSF – Granulocyte-Macrophage Colony Stimulating Factor
HEL – Hen egg white lysozyme
IC – Immune complex
IFN – Interferon
Ig – Immunoglobulin
IL – Interleukin
LOD – Logarithm of the odds
LPS – Lipopolysaccharide
ITAMs – Immunoreceptor tyrosine activation motifs
Mb – Mega base
mAb – Monoclonal antibody
MHC – Major histocompatibility complex
mDC – Myeloid dendritic cell
NZB – New Zealand Black
NZM – New Zealand Mixed
NZW – New Zealand White
ODN – Oligodeoxynucleotide
pDC – Plasmacytoid dendritic cell
PI – Propidium iodide
RBC – Red blood cell
qRT-PCR – Quantitative real-time polymerase chain reaction
sHEL – Soluble hen egg white lysozyme
ssDNA – Single stranded DNA
SLE – Systemic lupus erythematosus
SNP – Single nucleotide polymorphism
T – T lymphocyte
Tfh – T follicular helper cells
Tg – Transgene/transgenic
Th1 – T helper 1 cells
Th2 – T helper 2 cells
TLR – Toll-like receptor
T_{reg} – Regulatory T cell
TNF-α – Tumour necrosis factor alpha
TNFSF4 – TNF superfamily gene 4
Yaa – Y-linked autoimmune accelerator
Chapter 1

Introduction

1.1 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic multi-organ autoimmune disease that is characterized by the production of antibodies directed against nuclear antigens (1, 2). These autoantibodies (autoAb) bind to their cognate antigens, resulting in the formation of immune complexes (IC) that deposit in various organs such as the skin, joints, brain, heart, and kidney. This leads to activation of the complement system, resulting in inflammation and tissue damage (3, 4).

1.1.1 Genetic factors in SLE

Genetic factors are believed to play a significant role in the pathogenesis of lupus. SLE is a complex genetic disease in which multiple genetic polymorphisms, each of which contributes a relatively small increased risk, act in concert to produce the disease phenotype. Although genetic investigations such as genome wide association studies have been useful in identifying genetic polymorphisms that confer an increased risk for SLE, the precise alleles that are associated with SLE and the mechanisms by which they act to promote disease remain to be identified. In this context, studies of genetically modified or spontaneously arising lupus-prone mouse strains have been extremely helpful in providing a conceptual framework for lupus pathogenesis. These studies indicate that the genetic modifications that promote lupus can be classified into four groups (Table 1.1): 1) Those that promote impaired clearance of, or an abnormal response to, apoptotic debris; 2) those that alter the strength or nature of
signals delivered to B and/or T cells, resulting in abnormal stimulation of autoreactive lymphocytes; 3) those that lead to impaired apoptosis and/or increased survival of autoreactive lymphocytes; and 4) those that promote end organ damage (5, 6). In addition, study of spontaneously arising lupus-prone mouse strains has provided insight into how the individual genetic loci interact with each other to produce clinical disease. These studies have been greatly aided by primer-assisted breeding, the so-called ‘speed congenic’ technique that has enabled the rapid generation of congenic mouse strains in which a chromosomal region linked to the lupus phenotype is introgressed onto a well-defined, lupus-resistant (usually C57BL/6 (B6)) genetic background (7). By producing mice with combinations of susceptibility alleles, the genetic interactions between susceptibility loci can be examined.
Table 1.1. Proposed mechanisms and candidate genes implicated to promote SLE. (Adapted from Ref (60)).

<table>
<thead>
<tr>
<th>Impaired clearance &amp; aberrant response to apoptotic debris</th>
<th>Knock-outs and transgenics</th>
<th>Mouse Candidate genes from congenic studies</th>
<th>Human Genome-wide and candidate gene studies</th>
</tr>
</thead>
</table>
| Complement & clearance                                     | C1qa 
DNaseI 
SAP 
MFG-E8 
MeRd, LXR 
Ro 
DNaseI 
Ppap3 | C1q, Marco | C1q,C2,C4A, C4B, CRP, C2, ATG5, TREX1 |
| TLR & IFN signaling                                         | TLR7 Tg, Tlr8-/ | TLR7 (Yaa), IRAK1, Ifi202 | IRAK1, IRF5/7/8, SPP1, MECP2, STAT4, TYK2, IFI1 |
| NF-κB signaling                                             | Tnp1/- | | TNFAIP3, TNIP1, IKBKE, PRKCB |
|                                                             | | | UBE2L3, SCL15A4 |

<table>
<thead>
<tr>
<th>Aberrant adoptive immune response</th>
<th>Antigen presentation</th>
<th>H-2</th>
<th>HLA-DR2/3, HLA class III genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cell signaling</td>
<td>CD22 +/-</td>
<td>SHP-1 me</td>
<td>BLK, LYN, CSK, FCGR2B</td>
</tr>
<tr>
<td>Lyn +/-</td>
<td>Fcgr2b</td>
<td>MSH5, BANK1, PRDM1</td>
<td></td>
</tr>
<tr>
<td>FcγRIIb +/-</td>
<td>CD84, Ly108</td>
<td>ETS1, IKZF1, IL-10, IL-21</td>
<td></td>
</tr>
<tr>
<td>CD19 Tg</td>
<td></td>
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</tr>
</tbody>
</table>

| T cell signaling                                           | G2A +/- | Ly108 (SLAM) | L9 (SLAM), PTPN22 |
| Roquin san/san | Pbx1 | STAT4, TNFSF4 |
| Ro52 +/- | | IL10, IL-21 |

| Other                                                      | CD45 E613R | Pdcd1 +/- | Pp65ShcA +/- | PDCD1 |

| Promote survival of auto-reactive lymphocytes              | Bim +/- | PTEN +/+ | Fas +/- | FAS |
|                                                           | Bcl-2 Tg | BAFF Tg | Fasl +/- | FASL |
|                                                           | IL-2Rβ +/- | CTLA-4 +/- | | |

| Promote end organ damage                                   | Kallikreins | | KLK1, KLK3, ITGAM, ACP5, FCGR3B, FCGR2A, FCGR3A |

3
1.2 Lupus-prone mouse models

Several murine models spontaneously develop lupus-like disease and have been widely used to investigate the cellular and genetic basis of SLE induction. These include: the MRL/lpr, and BXSB/Yaa, New Zealand (NZ) mouse strains (such as New Zealand Black (NZB), New Zealand White (NZW), an F1 hybrid of NZB and NZW (NZB/W F1), and recombinant inbred mouse strains with a mixture of genetic material from these 2 strains (eg. NZM2410)) strains (8). Each of these models develops a unique iteration of lupus-like disease, exhibiting a subset of symptoms resembling those in human SLE, including autoantibody production, lymphocyte activation and lupus nephritis (9, 10).

In the subsequent sections, I will focus on what has been learned from these spontaneous mouse models of SLE, as studies of these mice have led to the identification of a number of potential genetic polymorphisms, signaling pathways and mechanisms of interaction by which lupus susceptibility loci produce the lupus phenotype.

1.2.1 MRL/lpr mice

The MRL strain was generated from inter breeding of several mouse strains, with the majority of the MRL genome deriving from the LG/J strain and minor contributions from C3H/Di, C57BL/6, and AKR/J strains (8). Characterization of MRL sub-strains revealed that one strain, named MRL/lpr, carries a single gene mutation that accelerates lupus-like disease. MRL/lpr mice are homozygous for the lymphoproliferation (lpr) mutation in the Fas receptor (FasR), which is located on chromosome 19. This mutation results in acceleration of the mild lupus-like autoimmunity seen in mice with the MRL background, leading to production of
high titers of various autoantibodies such as anti-ssDNA, anti-dsDNA, and anti-Sm antibodies, as well as rheumatoid factors. These autoantibodies result in the development of severe lupus nephritis and death from fulminant renal failure in approximately 50% of the mice by 5 months of age (9, 11, 12). In addition, these mice develop a marked accumulation of double negative (CD4\(^{-}\)CD8\(^{-}\)) B220\(^{+}\) T cells that results in the development of massive splenomegaly and lymphadenopathy (9, 13). Both males and females are significantly affected in the MRL/lpr strain.

1.2.1.1 Genetic associations/mechanisms associated with the development of lupus in MRL/lpr mouse strain

The FasR, which belongs to the tumor necrosis factor receptor (TNF-R) family, induces apoptosis upon binding its ligand FasL (14). In MRL/lpr mice, the genetic polymorphism encoding lpr leads to a truncated non-functional transcript of the Fas gene (15) and both the B and T cells from MRL/lpr mice have a defect in Fas-mediated apoptosis (16). Fas-mediated apoptosis plays an important role in the removal of germinal center B cells that have lost specificity for the immunizing antigen, such as those that have acquired autoreactivity, and studies show that B cell tolerance mechanisms are defective in MRL/lpr mice (17, 18). Activated T cells are also subjected to Fas-mediated apoptosis once they are no longer needed. In MRL/lpr mice, the marked expansion of CD4\(^{+}\)CD8\(^{-}\) T cells has been shown to be due to accumulation of ‘exhausted’ CD8\(^{+}\) T cells that have lost expression of CD8 (19-21).

A mutation in the Fas ligand gene gld (generalized lymphoproliferative disease) has also been shown to lead to an autoimmune disorder similar to the lpr mutation, with abnormal
survival and activation of autoreactive B cells and T cells (22), pointing out the importance of the Fas pathway in the prevention of lupus-like autoimmunity. Studies of the interaction between genetic background and the lpr mutation have demonstrated that although the lpr mutation promotes ANA production in non-lupus-prone mouse strains such as C3H/HeJ and C57BL/6J, it is not sufficient to cause nephritis in these strains (23). Therefore, MRL background genes also play an important role in promoting the development of disease in this mouse model.

Lupus susceptibility loci in MRL/lpr mice have been identified using F2 or N2 genetic crosses of MRL/lpr mice with various mouse strains, and genetic loci have been found on chromosomes 1, 4, 5, 7 and 10, that promote disease (9). Currently, there is limited data on the candidate genes within these loci. B6 congeneric mice, with a portion of MRL chromosome 1, have altered FcγRIIB and FcγRIII expression, which has been shown to promote development of GN in the presence of the lpr gene defect (24). A study of (MRL-Faslpr × C57BL/6-Faslpr) F2 mice revealed four susceptibility loci, Lmb1–Lmb4, that were linked to production of anti-dsDNA autoantibodies and glomerulonephritis (25). Studies of the Lmb3 lupus susceptibility locus on MRL-lpr chromosome 7, have shown that this locus leads to significantly enhanced T cell proliferation, as well as, anti-dsDNA autoantibody production and GN (9, 26). This enhanced proliferation was recently shown to be due to the presence of a functional coronin-1A gene in MRL mice. In B6 mice there is a single nonsense mutation in the coronin-1A gene, which ameliorates the autoimmune disease when crossed onto MRL/lpr background (27). Coronin-1A binds F-actin and prevents its assembly and depolymerization. The mutation in coronin-1A in B6 mice leads to increased T cell apoptosis and reduced T cell activation and
proliferation, suggesting that amelioration of disease by this mutation is mediated through impaired activation and/or increase apoptosis of autoreactive T cells.

In human lupus, defects in the Fas signaling pathway are rarely seen. However, individuals with defects in this pathway (mostly due to dominant negative mutations in FasR) have been described and have a similar syndrome, termed the autoimmune lymphoproliferative syndrome, to that observed in MRL/lpr mice. Although these individuals have lymphoid expansion due to accumulation of CD4^-CD8^- T cells and develop autoimmunity, the severity of these phenotypes is highly variable even within the same pedigree, highlighting the requirement for interactions with other background genes for expression of the disease phenotype (28).

1.2.2 BXSB mice

The BXSB mouse strain is a recombinant inbred cross between the C57BL/6 and SB/Le mouse strains (9, 29). In contrast to other lupus-prone mouse strains, the disease is more severe in male than female BXSB mice. Lupus-like disease in these mice is characterized by: anti-nuclear, -erythrocyte, -cardiolipin, and -platelet antibody production, enlarged secondary lymphoid organs, and severe immune complex-mediated GN. Acceleration of disease in male mice has been shown to be due to the presence of the Y-linked autoimmune accelerator (Yaa) locus, which results in an increased rate of mortality in male versus female mice due to proliferative GN. Approximately 50% of males die by 5 months of age, whereas this takes ~14 months in female mice (30).
1.2.2.1 Genetic associations/mechanisms associated with the development of lupus in the BXSB mouse strain

It has been demonstrated that the Yaa locus is not sufficient to induce the development of disease in non-autoimmune-prone mouse strains, such as the CBA/J or C57BL/6 strains (31, 32), but that it can significantly augment disease onset in other spontaneous lupus-prone mouse strains including the New Zealand Black (NZB), New Zealand White (NZW) and MRL/lpr strains (31, 33). Thus, it has been suggested that the Yaa locus acts together with other lupus susceptibility genes to potentiate the disease.

It is now known that a translocation of the telomeric end of the X chromosome to the Y chromosome is responsible for the Yaa locus. This results in the duplication of at least 16 genes, which consequently leads to a two-fold increase in the expression of many of these genes (34, 35). Among these genes is Toll-like Receptor 7 (Tlr7), which has been shown to be involved in augmenting lymphocyte signalling and accelerating lupus pathogenesis (35, 36). There also appears to be a minor influence from other genes located within the Yaa interval, as knock out of Tlr7 does not reduce all Yaa-induced phenotypes. For example, in Yaa+ mice, there is still elevated autoantibody production in Tlr7 knockout mice compared to Tlr7 sufficient mice (37, 38).

As mentioned above, B6 mice with the Yaa locus do not develop lupus, suggesting the requirement for additional lupus susceptibility genes from the BXSB background for expression of disease. Several mapping studies have been performed leading to the identification of multiple lupus susceptibility loci in BXSB mice on chromosomes 1, 3, and 13 (39, 40). At least four susceptibility loci, termed Bxs1-4, are located on chromosome 1, and studies using congeneric mouse models have confirmed that these loci are linked to nephritis.
and anti-dsDNA autoantibody production (41). Of these, Bxs3 overlaps with the Sle1 locus found on chromosome 1 in the NZM2410 lupus-prone mouse strain and seems to have the strongest effect on disease development. Although Bxs5, which is located on chromosome 3, modulates disease, it appears to act as a suppressor locus (42), whereas Bxs6, located on chromosome 13, has been linked to increased production of antibodies to serum glycoprotein subunit 70 (gp70), and gp70 immune complex (gp70IC), which are thought to promote renal disease (43). The presence of the Yaa locus significantly increases Bxs6-mediated disease activity, resulting in increased nephritis due to upregulated production of anti-gp70 autoantibody and elevated levels of gp70 IC deposition in glomeruli (30). Bxs6 overlaps with two genetic loci discovered in other lupus-prone mouse strains, NZW-derived Sgp3 (44) and 129-derived Gv1 (45), which have also been shown to play a role in the regulation of serum gp70 levels.

A single candidate gene named Marco (macrophage receptor with collagenous structure) has been identified on chromosome 1 in BXSB mice. Marco is an innate scavenger receptor that plays a role in the clearance of apoptotic debris. In BXSB mice, lower RNA and protein levels lead to defective clearance of apoptotic cells by macrophages, which based upon studies of mice with induced mutations, is thought to promote the development of lupus-like autoimmunity (46).

1.2.3 NZ mice

The NZ mouse strains include the NZ Black (NZB), NZ White (NZW), (NZB x NZW)F1 cross (NZB/W), and recombinant NZ Mixed (NZM) inbred lines derived by crossing NZB and NZW mouse strains, of which NZM2410 and NZM2328 are the best characterized
These strains are thought to closely mimic the human disease, with a similar female sex bias (~6:1 ratio) for disease development. Consequently, numerous studies have used these mouse strains to investigate the pathogenesis of lupus (5). The NZB strain spontaneously develops a lupus-like autoimmune disease characterized by production of anti-ssDNA and -red blood cell antibodies leading to hemolytic anemia at 6 to 8 months and mild GN at 12 months of age, with the mice typically dying around 16 months of age (reviewed in (8)). This mouse strain appears to possess all of the genes necessary to develop lupus nephritis except a permissive MHC haplotype, as MHC congenic NZB.H2b and NZB.H2bm12 mouse strains spontaneously develop severe kidney disease (48).

Although the NZW mouse does not develop autoimmunity, it was found that F1 crosses between NZB and NZW mice developed more severe kidney disease when compared to the parental strains. This is likely due to the elevated levels of IgG serum antinuclear autoantibodies (ANA), in particular anti-dsDNA Abs, which has been shown to deposit in the kidneys of these mice around 5-6 months of age, resulting in the development of proteinuria and fatal GN. Approximately 50% of the mice die from kidney disease by 8.5 months of age (49).

Backcrossing of the NZB/W F1 and NZW strains, followed by brother-sister mating, has been used to generate 27 fully inbred mouse strains with both NZB and NZW genetic material, labeled NZM (50). Among the NZM mouse strains, the NZM2410 and NZM2328 mouse strains have been extensively examined and characterized. As in NZB/W mice, both NZM2410 and NZM2328 mouse strains demonstrate high titer anti-dsDNA production and severe GN resulting in 50% mortality at 5 to 6 months of age; however, NZM2410 has a weaker gender bias as both male and female mice develop the disease (50-52).
1.2.3.1 Genetic associations/mechanisms associated with the development of lupus in NZ mouse strains

Several groups, including our laboratory, have conducted extensive mapping studies of various NZ mouse strains to identify the susceptibility loci and genetic polymorphisms responsible for disease development. Wakeland’s group was the first to conduct a mapping study of the NZM2410 mice, leading to the identification of three major lupus susceptibility loci (Sle1, Sle2, and Sle3) that were linked to anti-dsDNA autoAb production and the development of severe GN in this mouse strain (51). To determine how each susceptibility locus in the NZM2410 mouse contributed to autoimmunity, a series of congenic mice was generated by this group, in which these loci were introgressed onto the B6 background (53-59). Sle1, located on chromosome 1, appeared to play a critical role in the loss of tolerance to nuclear Ags, impacting on both T and B cell function and resulting in the production of antinuclear autoantibodies (59). Sle2, located on chromosome 4, led to B cell hyperactivity, polyclonal activation, and expansion of both splenic and peritoneal CD5+ B1a cells as well as elevated serum levels of IgM, but did not induce any autoimmune pathology (53, 60). Similarly, Sle3, located on chromosome 7, was not by itself associated with autoimmune disease, although these mice demonstrated expansion and proliferation of CD4+ T cells with reduced activation-induced T cell death (61). Although each of these three loci on their own did not lead to the development of a severe autoimmune phenotype, subsequent studies examining mice with two or three of these congenic intervals showed that epistatic interactions resulted in increasingly severe disease, with tri-congenic mice demonstrating a phenotype similar to the parental strain.
A surprising finding of these NZM mapping studies was that the majority of genetic loci that promote disease in these mice are derived from the NZW parent. Since NZW mice do not have an autoimmune phenotype, this raised the question of what prevents the development of autoimmune disease in this mouse strain. Subsequent mapping studies showed the presence of several NZW-derived suppressor loci, the strongest of which, Sles1, was closely linked to the major histocompatibility complex (MHC) class II locus H-2^z (62). Study of the Sles1 locus showed that it interacts with the Sle1 locus, ameliorating the abnormal B and T cell activation, autoantibody production and development of renal disease (63).

Early mapping studies done in NZB/W mice indicated that multiple genes from the NZB parent contributed to the severity of disease in this mouse strain. These genes mapped to chromosomes 1, 4, 7, 10, 13, and 19, and appeared to act in concert to induce severe renal disease (49, 64-66). Subsequent studies, by our group as well as others, have confirmed the presence of susceptibility loci that promote the generation of autoantibodies and/or nephritis on NZB chromosomes 1, 4, 7, and 13 (5, 67, 68), and congenic mice with intervals corresponding to these loci have been produced. However, only mice with intervals derived from NZB chromosome (c) 1 or 13 spontaneously develop lupus-like autoimmunity.

1.2.4 Chromosome 1 congenic mouse strains

Although autoimmune disease in each lupus-prone mouse strain arises spontaneously from the interactions between several different genetic loci, some of which facilitate and some of which inhibit disease, there is significant overlap of these loci between the different lupus-prone strains, particularly for loci contained on chromosomes 1, 4, 7 and 13 (reviewed in (6, 67-
Generation of congenic mouse strains, in which a chromosomal region linked to the lupus-phenotype is introgressed onto a non-autoimmune background, has played an important role in the identification of these genetic loci and the mechanisms by which they act to promote disease (70). Of the 35 murine susceptibility loci that have been identified on these chromosomes in 6 different lupus-prone mouse strains, 6 have been mapped to chromosome 1, indicating the importance of this chromosomal region in the generation of the lupus phenotype (10). Multiple susceptibility loci located at the telomeric end of chromosome 1 in lupus-prone mouse strains, including NZB, NZM2410, NZM2328, 129, MRL/lpr and BXSB mice, have been repeatedly mapped and linked to development of autoantibodies and/or nephritis (39, 51, 70, 71). The telomeric region of c1 is synergic to human c1q23-42, a region confirmed to have several associations with human lupus. To identify the susceptibility loci that contribute to the development of disease in this region, several congenic mouse strains, including B6.Sle1 from NZM2410 mice (interval of NZW origin) (72), B6.NZBc1 and B6.Nba2 from NZB mice (73, 74), B6.MRLc1 from MRL mice (71), and B6.Bxs1-4 from BXSB mice (41), have been generated. Although the MRL and BXSB c1 congenic mouse strains demonstrated autoantibody production and have intervals that overlap with those studied in NZ mice, little is known about the candidate genes in these mice. Therefore in the subsequent sections I will focus on NZ-derived c1 congenic mice as they are more relevant to my thesis.

1.2.4.1 NZM-derived chromosome 1 congenic mouse strains

The best characterized of the c1 congenic mouse strains is the B6.Sle1 strain, in which susceptibility loci derived from the NZM2410 154-197 Mb interval of chromosome 1 have been introgressed onto the B6 mouse background. Studies of mixed hematopoietic chimeric mice with a mixture of B6 and B6.Sle1 bone marrow indicated that intrinsic B and T cell functional
defects contribute to the loss of tolerance to chromatin with production of IgG anti-chromatin autoAbs and priming of autoreactive T cells to the histone epitopes on chromatin (57, 75). Through generation of subcongenic mice, in which each mouse carries different portions of the Sle1 locus, it was later shown that four non-overlapping loci within Sle1, termed Sle1a, Sle1b, Sle1c and Sle1d, independently contribute to the development of the autoimmune phenotype in these mice (76-82).

Sle1a (localized to the 168.3-173 Mb region of the Sle1 locus) was shown to have the strongest association with lupus nephritis in the NZM2410 mouse model. This interval is associated with spontaneous priming of T cells to nucleosomes and reduced proportions of CD4+Foxp3+ regulatory T cells (82). Two independent loci, Sle1a1 and Sle1a2, comprise the Sle1a locus and both intervals are required for complete expression of the autoimmune T cell phenotype (82). Recent studies have shown that the Sle1a1 phenotype likely results from the expression of a novel splice isoform of the Pbx-1 gene, named Pbx1d. Pbx-1 expression is reported to increase in activated/memory CD4+ T cells (83). Through overexpression of Pbx-1d in Jurkat T cells, it was shown that Pbx1-d induces the expression of genes related to T cell activation and Th17 cell differentiation. Since Pbx1 is the only relevant gene in the Sle1a1 interval, it was suggested that the enhanced proportion of activated and autoreactive CD4+ T cells and decreased Treg populations seen in Sle1a1 mice likely results from the expression of Pbx1-d isoforms in activated/memory T cells. The genetic function of Sle1a2 is currently unknown.

Sle1b is localized to a 1 Mb interval extending from 173-174 Mb within the Sle1 locus. This region demonstrates the strongest association within the Sle1 locus with high titer anti-nuclear antibody (ANA) production (84, 85). Extensive polymorphism of the SLAM/CD2 gene
family (including Cd244, Cd229, Cs1, Cd48, Cd150, Ly108 and Cd84) has been reported in the Sle1b region (85). Several of these molecules were shown to be differentially expressed in T and/or B cells, with Ly108 being the most studied of these. Differential expression of Ly108 splice isoforms by B6 and NZM2410 alleles has been shown to impact on B cell deletion and anergy induction, as well as the T cell activation and differentiation to IFN-γ producing cells in B6.Sle1b mice (84-86). Recently, polymorphisms in both CD84 and Ly108 in B6.Sle1b mice were shown to lead to attenuated BCR signaling, resulting in reduced apoptosis, attenuated B cell–T cell interactions, impaired GC tolerance mechanisms, and the generation of ANA-producing cells (87).

Sle1c, another sub-locus of Sle1 that is located in the NZM 190-197 Mb interval, is also associated with GN and anti-chromatin IgG production, but at lower penetrance than is observed for the Sle1b locus (72). Further studies revealed that this locus contained two sub-loci, Sle1c1 and Slelc2. Within the Sle1c1 interval, a single nucleotide polymorphism (SNP) has been identified in the Cr2 gene, which encodes the complement receptor type 2. Studies have shown that this receptor type acts as a B cell co-receptor, potentially contributing to the Sle1c1 phenotype by impacting on germinal center tolerance mechanisms (61, 78). Analysis of B6.Sle1c2 CD4+ T cell function revealed the presence of an intrinsic T cell defect, resulting in the accumulation of activated T cells and a decreased proportion of regulatory T cells in this congenic mouse strain (61). These mice also exhibited a significant expansion of IFN-γ expressing T cells (88).

The fourth locus, Sle1d, located between Sle1b and Sle1c2, increases the severity of GN when B6.Sle1d mice are crossed with NZW mice (72); however, the candidate gene(s) within this region has not been identified.
1.2.4.2 NZB-derived chromosome 1 congenic mouse strains

Congenic mouse models with NZB chromosome 1 intervals have been generated independently by our laboratory and others. B6.Nba2 congenic mice, originally generated by Kotzin’s group, carry a homozygous 155 to 194 Mb interval from NZB chromosome 1. This interval leads to the development of a lupus-like disease with elevated serum levels of ANAs, lymphadenopathy, splenomegaly, IgG immune complex deposition in the kidneys, and elevated levels of IFN-α in serum. However, B6.Nba2 congenic mice do not develop severe GN (65, 89, 90). Several candidate genes were proposed in this interval including the FcγR family, SLAM/CD2 family, and the IFN-inducible Ifi200 family of genes (encoding for the p200 family proteins), all of which are polymorphic in NZB as compared to B6 mice.

A number of subcongenic mouse strains with smaller intervals derived from the initial congenic interval containing the Nba2 lupus susceptibility locus were generated, denoted B6.Nba2-A (154.7-174.5Mb), B6.Nba2- A'B (169.1- 175.9Mb), B6.Nba2-B (172.8-175.9Mb), B6.Nba2-BC (172.8-194.1Mb), and B6.Nba2-C (174.5-194.1Mb) (91). B6.Nba2-A (154.7-174.5Mb) female mice harboring the NZB FcγR gene locus and B6.Nba2-B (172.8-175.9Mb) mice carrying the NZB Slam locus both developed detectable levels of ANAs. However, significantly higher levels of ANAs were found in the B6.Nba2-A'B mice that had both the NZB FcγR and the NZB Slam-family genetic loci, raising the possibility that these two loci work in tandem to promote autoantibody production (91, 92). B6.Nba2-A'B mice also produced increased levels of type I IFN. B6.Nba2-C subcongenic mice, with just the Ifi200 family of genes, developed neither ANAs nor type I IFN elevations. Consistent with a negligible effect of the polymorphisms in this gene family on the development of the autoimmune phenotype in B6.Nba2 mice, antibody production was not increased in B6.Nba2-BC as compared to
B6.Nba2-C mice. Although the authors of this study suggest that their findings confirm the role of the FcγR and SLAM loci in production of ANA and nephritis in B6.Nba2 congeneric mice, an important caveat to this work is that each of these intervals contains a number of additional genes that could contribute to the autoimmune phenotype, and therefore the findings do not prove that these genetic loci are indeed the relevant candidate genes in these intervals.

Our laboratory has also generated a number of NZB c1 congeneric mouse strains. Based upon the results of a mapping study that identified the 1 LOD (logarithm of the odds score) confidence interval for a genetic locus (loci) linked to B cell activation and autoantibody production on chromosome 1 in NZB mice, congeneric mice were produced with a homozygous NZB interval extending from 35 to 106 centimorgans (cM) (62-191 Mb) introgressed onto the B6 background (66). These mice, termed c1(35-106), produced high titers of IgG anti-ssDNA and –chromatin autoantibodies and developed moderate non-lethal renal disease (66, 93). In addition, increased spontaneous T and B cell activation and an increased number of germinal centers were noted. Studies of mixed hematopoietic chimeric mice with a mixture of c1(35-106) and tagged B6 bone marrow indicated the presence of intrinsic B and T cell functional defects in these mice, resulting in increased spontaneous activation of T and B cells, with enhanced recruitment of c1(35-106) B cells into germinal centers, as compared to their B6 counterparts (93).

Subsequently, to further localize the susceptibility loci on NZB chromosome 1, a series of subcongeneric mouse strains with smaller c1 intervals was produced (shown in Figure 1.1).
Figure 1.1 Genetic map of the c1 congenic mouse strains. Shown are the subcongenic lines generated from the original c1(35-106) congenic mouse strain. Thick and thin black lines denote NZB and B6 regions, respectively. The scale above shows the distance measured in megabases (Mb) from the centromere. The presumptive regions for each of the four NZB loci that modulate the autoimmune phenotype are highlighted with grey boxes. The borders of each interval measured in Mb are shown to the right of the figure together with the name for each congenic mouse strain, which is based upon the original borders defined in cM.
Congenic mice with NZB 100-106 cM intervals or 43-85 cM intervals did not produce autoantibodies, whereas mice with a 70-100 interval produced high titers of anti-dsDNA Abs and developed severe GN, leading to death of ~40% of the mice by 8 months of age (94). These findings localized the gene(s) that lead to autoantibody production to the 85-100 interval. Consistent with this inference, congenic mice with an NZB interval extending from 88 to 100 cM (168.3-179.8 Mb; c1(88 to 100)) produced anti-dsDNA antibodies and developed moderate non-lethal GN, suggesting that a gene or genes within the 70-88 region augments the severity of renal disease. Mice with the shortest NZB interval that was crossed onto the B6 background, which extends from 96 to 100 cM (170.8-179.8 Mb; c1(96-100)), had mild subclinical autoimmunity characterized by production of anti-ssDNA Abs in the absence of anti-dsDNA Abs or kidney disease. These findings indicate that there is a genetic locus (loci) within the 96-100 cM region that breaches tolerance to nuclear antigens, but that at least two additional loci within the 70-96 interval are required for full expression of the autoimmune phenotype in c1(70-100) mice.

Studies in the laboratory have further characterized the B and T cell functional abnormalities in c1(96-100) mice. To investigate the nature of the B cell tolerance abnormalities in these mice, soluble hen egg lysozyme (sHEL) and anti-HEL immunoglobulin (Ig) transgenes were crossed onto the c1(96-100) background. B cells from double transgenic c1(96-100) mice showed elevated expression of activation markers, increased recruitment into germinal centers, and significantly enhanced production of IgG and IgM anti-HEL autoantibodies, as compared to corresponding B6 B cells (N. Chang et al, manuscript in preparation). These findings suggest that there is a generalized breach of B cell anergy in these mice.
It is likely that other mechanisms of B cell tolerance in these mice are also defective. Studies of HEL double transgenic mice, performed in the laboratory, indicate that there is attenuated BCR signaling with reduced Ca\(^{2+}\) mobilization in the immature B cells of c1(96-100) as compared to B6 mice and that this is associated with reduced receptor editing and impaired apoptosis of the self-reactive B cell compartment. In this respect, the B cell defect in c1(96-100) mice is similar to that observed for B6.Sle1b mice, suggesting that it might arise from shared polymorphisms in the SLAM family. This remains to be confirmed, as there are some sequence differences in this locus between the NZB and NZM alleles.

Studies by our laboratory and others suggest that germinal center tolerance mechanisms may also be defective in c1(96-100) mice. It is possible that this defect is related to the SLAM polymorphisms in NZB mice, as previous work in the NZM mouse model suggests that these polymorphisms impact on germinal center tolerance (84). Altered expression of the inhibitory type II Fc\(\gamma\)R (Fc\(\gamma\)RIIB), due to a polymorphism in the promoter region of the Fcgr2b gene, has also been proposed to contribute to the altered germinal center tolerance and increased production of autoantibodies observed in NZB mice (95, 96). However, studies of Fc\(\gamma\)RIIb knockout mice suggest that absence of this receptor leads to enhanced development of plasma cells and increased production of autoantibodies, but has little impact on other B cell tolerance mechanisms (97). Notably, comparison of the immunologic phenotype observed in c1(96-100) mice, which have an interval containing both the Fc\(\gamma\)R and SLAM loci, with published results of mice with the B6.Nba2 A’B and B intervals, suggests that these mice have the same phenotype as B mice, and that our c1(88-100) mice have the same phenotype as the A’B mice. These findings suggest that the enhanced autoantibody production observed in B6.Nba2A’B mice (and c1(88-100) mice) is not due to the Fc\(\gamma\)R locus, as previously proposed, but another locus in this
interval. Experiments outlined in this thesis provide insight into this locus and will be outlined in detail later.

In addition to B cell defects, c1(96-100) mice also demonstrate altered T cell function. T cells from young c1(96-100) mice have a decreased threshold for IFN-γ production and T cell proliferation following stimulation with anti-CD3 when compared with B6 control T cells. It is possible that this functional alteration arises from polymorphisms in the SLAM locus, particularly in Ly108, as previous work has shown that the absence of the Ly108-H1 splice variant in NZM mice leads to enhanced differentiation of IFN-γ producing T cells (86).

Comparison of subcongenic mouse strains with longer intervals that included the c1(96-100) region showed an increased number of splenic germinal centers as compared to c1(96-100) mice. The increases in the number of splenic germinal centers observed in c1(88-100) and c1(70-100) mice were similar, and roughly paralleled increases in the extent of chronic T cell activation, as indicated by elevated proportions of recently activated (CD69+) and memory/effector (CD44hiCD62Llo) T cells in these mice (94). These findings imply that there is at least one locus in the 88-96 interval that is involved in qualitative or quantitative aspects of T cell help. This interval overlaps with the Sle1a susceptibility locus, previously identified in NZM2410 mice (Figure 1.2). Although this interval in NZM mice is derived from the NZW parent, 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, suggesting that they may share susceptibility loci with NZB mice in this region (72, 98). This possibility was addressed by investigations in this thesis.
It is likely that there is another susceptibility locus located in the 70-88 cM interval (125.6 - 168.3Mb) of NZB mice. Addition of this region to the 88-100 cM interval resulted in increases in B cell activation, autoantibody titers, immunoglobulin deposition in the kidney, renal disease, and mortality. Experiments examining the immune mechanisms leading to the increased disease severity in these mice are outlined in this thesis.
Figure 1.2. Comparison of validated lupus susceptibility loci on mouse chromosome 1 in different mouse strains. The congenic intervals corresponding to each locus are denoted by boxes with their size in Mb (millions of base pairs) shown relative to the scale on the top. The lupus prone mouse strain of origin is shown on the right, and the name of each locus is indicated on the left of the box. The grey and black boxes denote congenic mice with NZB intervals, while the white boxes indicated intervals derived from NZM2410 mice which are of NZW parental origin.
1.3 The SLAM/CD2 family of receptors

1.3.1 General characteristics

The signaling lymphocyte activation molecule (SLAM) family constitutes a group of receptors that regulate the activation and differentiation of a wide variety of cell types involved in innate and adaptive immune responses (99-102). The SLAM family of receptors belongs to the CD2 subset of the immunoglobulin superfamily and has nine distinct members: SLAMF1 (CD150, SLAM), SLAMF2 (CD48), SLAMF3 (CD229, LY9), SLAMF4 (CD244, 2B4), SLAMF5 (CD84), SLAMF6 (CD352, NTB-A (NK-T-B-antigen) in humans or Ly108 in mice), SLAMF7 (CD319, Cs1 or CRACC (CD2-like receptor activating cytotoxic cells)), SLAMF8 (CD353 or BLAME (B lymphocyte activator macrophage expressed)) and SLAMF9 (CD84-H1). The genes that encode seven of the SLAM family members, SLAMF1-SLAMF7 (the SLAM/CD2 cluster) are located within a 400-500 kilobase (kb) genomic segment on human chromosome 1q23 or mouse chromosome 1H3 (99, 103). The other two remaining SLAM family members (SLAMF8 and SLAMF9) are located outside of the SLAM/CD2 cluster (roughly 1 Mb centromeric to the SLAM/CD2 cluster) (99, 104). The SLAM molecules are ubiquitously expressed on variety of immune cells including different subsets of T and B lymphocytes, NK and NKT cells, monocytes, macrophages, DCs, pDCs, platelets, granulocytes, and hematopoietic stem and progenitor cells, but the precise family members expressed and their downstream adapters vary with each cell type (Table 1.2) (99, 103, 105).
Table 1.2: Expression pattern, function, and signal transduction effector molecules for the SLAM receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>cellular distribution</th>
<th>Function</th>
<th>Effectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLAMF1(CD150)</td>
<td>SLAM (CD150), Measles virus</td>
<td>Thymocytes (highest DP), naive B cells, memory T cells, in vitro activated T and B cells, DCs, platelets, HSCs</td>
<td>IL-4 secretion by CD4+ T cells, IL-12, TNF-α production by macrophages</td>
<td>SAP, EAT-2, Fyn, Akt, SHP-1, SHP-2, Dok1, Dok2, SHC, Ras-GAP, NF-κB</td>
</tr>
<tr>
<td>SLAMF2(CD48)</td>
<td>CD244(2B4)</td>
<td>NK cells, γδ T cells, memory CD8+ T cells, monocytes, basophils, eosinophils</td>
<td>NK cell cytokine secretion, cytotoxicity. Immune synapse formation in CD8+ T cells. May also signal through its ligand CD48</td>
<td>SAP, EAT-2 Lck, Fyn,</td>
</tr>
<tr>
<td>SLAMF3 (CD229, Ly9)</td>
<td>Ly9(CD229)</td>
<td>Thymocytes, T, Thh, NKT, B, NK (low), macrophages, DC</td>
<td>Negative regulator of TcR signaling. Minimal phenotype of ly9−/− mice</td>
<td>SAP, EAT-2 ERT Fyn, LAT Vav1, CBL, PI3K, Ca2+ Flux, ERK1/2, 3BP2, CSK, SHP-1, SHP-2, SAP, EAT-2</td>
</tr>
<tr>
<td>SLAMF4 (2B4, CD244)</td>
<td>CD48</td>
<td>MPP, NK, γδ, activated CD8+ T, CD8 iELs, monocytes, basophils, eosinophils</td>
<td>NK cell cytokine secretion, cytotoxicity. Immune synapse formation in CD8+ T cells</td>
<td>SAP, EAT-2, Fyn, Grb2, ERK SHP-2, SAP, EAT-2</td>
</tr>
<tr>
<td>SLAMF5 (CD84)</td>
<td>CD84</td>
<td>Most thymocytes, HSCs, NK, NKT, B, T, monocytes, platelets, DC, eosinophils, DCs, neutrophils, basophils, eosinophils, Thh</td>
<td>? T cell proliferation, cytokine secretion</td>
<td>SAP, EAT-2, SHP-1, SHP-2</td>
</tr>
<tr>
<td>SLAMF6 (CD352)</td>
<td>Human: NTB-A, Mouse: Ly108</td>
<td>NK cells, T cells, NKT cells, Thh cells, B cells, eosinophils</td>
<td>IL-4 and IFN-γ production by CD4+ T cells. Neutrophils function: IL-12, IL-6, TNF-α production</td>
<td>SAP, EAT-2, Fyn, Vav1, CBL, Ca2+ Flux, SHP-1</td>
</tr>
<tr>
<td>SLAMF7 (CD319, CRACC, CS1)</td>
<td>CRACC (CD319)</td>
<td>NK, B, mature DC, plasma cells, activated CD4 and CD8+ T</td>
<td>NK cell cytotoxicity cytokine production by CD4+ T cells</td>
<td>EAT-2, Fyn, PLCγ1, PLCγ2, PI3K, Ca2+ Flux, CSK, SHP-1, SHP-2</td>
</tr>
<tr>
<td>SLAMF8, BLAME</td>
<td>SLAMF8</td>
<td>Macrophage, DC</td>
<td>Negative regulator of macrophage function</td>
<td>No data</td>
</tr>
<tr>
<td>SLAMF9, SF2001 CD84-H1</td>
<td>SLAMF9?</td>
<td>T cells, B cells, monocytes, DC</td>
<td>No data</td>
<td>No data</td>
</tr>
</tbody>
</table>

Abbreviations: DC: dendritic cell; HSCs: hematopoietic stem cells; iELs, intraepithelial lymphocytes; MPP: multipotent hematopoietic progenitors; Thh: T follicular helper cell; CBL: casitas B-lineage lymphoma; CSK: COOH-terminal Src kinase; Dok1/2: Docking protein ½; Grb-2: growth factor receptor–bound protein 2; LAT: linker for activated T cells; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K: phosphoinositide 3-kinases; PLCγ: phospholipase Cγ; SAP: SLAM-associated protein; SHC: Src homology 2 containing; SHIP: SH2-containing inositol polyphosphate 5-phosphatase; SHP-1/2: SH2 domain–containing phosphatase ½.
The SLAM family of receptors are type I glycoproteins. In addition to two extracellular immunoglobulin (Ig)-like domains, each receptor has a cytoplasmic signaling domain that bears multiple tyrosine-based activation motifs which, through linkage with members of the SLAM-associated protein (SAP) family of signaling adaptors, convey either activating or inhibitory signals (102). SLAMF1, 2B4, CD84, Ly108, CRACC and Ly9 all share similar extracellular and cytoplasmic domains (Figure 1.3). These SLAM molecules all have intracellular tyrosine based switch motifs (ITSMs; consensus sequence TxYxxV/I) in their cytoplasmic tails, which are crucial for signaling and transmit either activating or inhibitory signals via associations with intracellular SH2 domain-containing adaptor molecules. With the exception of 2B4, all members of this core SLAM family interact in a homophilic manner in a head-to-head conformation, and therefore act as self-ligands (106-111). Phosphorylation of Y residues within the ITSM is triggered by these homophilic interactions, which consequently act as a docking site for intracellular adaptor molecules and enzymes bearing SH2 domains, such as SHP-2, SHP-1, Csk, and SHIP-1. However, the adaptor molecules SLAM-associated protein (SAP), EWS/FLI activated transcript-2 (EAT-2) and EAT-2-related transducer (ERT) have a particularly high affinity for this unique motif (99, 112). Genes encoding EAT-2 and ERT, the latter a pseudogene in humans, are located in the vicinity of the SLAM locus on chromosome 1, while the SAP gene has been mapped to the human and murine X chromosome.
Figure 1.3. Structural representation of the six core SLAM family members. The typical SLAM receptor consists of an extra-cellular region that contains an I- like V- and C2-like domain. The cytoplasmic domain contains 2-4 ITSMs that are binding sites for SAP, as well as other SH2 domain-containing proteins such as EAT-2. A number of splice variants exist for these receptors that can vary the number of ITSMs. Adapted from (105) with the author’s permission.
1.3.2 SLAM family polymorphisms and lupus

Extensive polymorphisms in many of the SLAM/CD2 genes have been identified and linked to autoimmune diseases in humans and mice (113). Several lupus-susceptible mouse strains have been reported to have polymorphisms in the SLAM family. As outlined previously, the Sle1b locus contains the SLAM locus, and this very polymorphic cluster of genes has been identified as potential candidate genes in this region (85). Subsequent experiments have largely focused upon the role of Ly108/SLAMF6 in this phenotype, where differences in the expression of several splice variants of this gene have been noted. Ly108 was initially found to have two isoforms resulting from alternate splicing (114). B6 mice express predominantly the Ly108.2 isoform, whereas NZM2410 mice express higher levels of the Ly108.1 isoform (85). NZB, NZW, BXSB, 129, and MRL mice were all shown to have similar SLAM alleles to NZM2410 mice, suggesting that the SLAM locus acts as a lupus susceptibility locus in multiple strains of mice.

Functional differences in the two Ly108 isoforms were found after transfection of a mouse T cell line with the Ly108.1 isoform resulted in stronger SAP-dependent protein tyrosine phosphorylation of Vav-1 and c-Cbl as compared to the Ly108.2 isoform (115). This is in keeping with the observation that T cells derived from B6.Sle1b exhibit increased TCR-induced Ca\(^{2+}\) flux (85) and more robust proliferation (86) when compared to B6 controls. However, this differed in the B cell compartment, where transfection of the B cell WEHI-231 line with Ly108.1 isoform resulted in reduced Ca\(^{2+}\) influx upon IgM crosslinking, reduced cell death, and lower RAG expression (84). Similar phenotypes were seen when the immature B cell compartment was examined in the B6.Sle1b mouse strain, with immature B cells demonstrating muted signal transduction downstream of the BCR, reduced Ca\(^{2+}\) flux,
impaired RAG re-expression, and reduced deletion, as compared to their B6 counterparts (84). Recently, Terhorst’s group reported a third isoform of Ly108, named Ly108-H1, expressed in the cells of the B6 mouse strain, but absent in B6.Sle1b mice. Introduction of a transgene expressing the Ly108-H1 encoding allele onto the B6.Sle1b background prevented the development of lupus in this mouse strain (86). Together, these observations demonstrate that genetic variation in Ly108 may be an important factor in the induction of lupus in B6.Sle1b mice through its effects on B and T cell function and tolerance.

The other polymorphic variants of the SLAM family receptors in the B6.Sle1b mouse strain are located in the exon regions of 2B4, Ly9, CRACC, and CD84 (85). The consequence of these polymorphisms and their contribution to the autoimmune phenotype in various lupus mouse models has yet to be determined. It has been proposed that a change in the amino acid sequence in the binding domains of these receptors may result in changes to their affinity for their ligands and, as a consequence, may affect the activation of different downstream signal transduction pathways mediated by these receptors in different immune cells (116).

As previously outlined, while NZB mice are thought to share many of the same polymorphisms in the SLAM locus that are seen in NZM mice, some differences have been reported. Consequently, it is not known to what extent the findings observed in B6.Sle1b mice will be replicated in NZB congenic mice.
1.3.3 The SLAM-associated protein (SAP) family adaptors

The SAP family of adaptor molecules includes SAP (encoded by Sh2d1a), EAT-2 (encoded by Sh2d1b1) and ERT (encoded by Sh2d1b2). As outlined previously, following SLAM engagement, the SAP family of adaptors binds to the phosphorylated ITSM (117) through the SH2 domain. Seven of the SLAM receptors have been shown to associate with either SAP and/or EAT-2/ERT (see Table 1.2) (117, 118). Which adaptor binds to the receptor is dictated both by the SLAM molecule that is being engaged and by the cell type that it is expressed in. SAP adaptors are only expressed in immune cells. SAP is found in T cells, NK cells, NK-T cells, and B cells (germinal center and memory B cells) (119, 120), while EAT-2 is expressed in NK cells, DCs, and macrophages. ERT has only been found in mouse NK cells.

It was initially reported that SAP promoted cell activation by blocking the association of the SLAM receptors with inhibitory transduction molecules, such as SH2 domain-containing protein tyrosine phosphatase-1 (SHP-1), SH2 domain-containing protein tyrosine phosphatase-2 (SHP-2), SH2 domain-containing inositol-5-phosphatase (SHIP-1) and the inhibitory kinase Csk (121). Subsequent work identified a novel mechanism of SAP adaptor function, whereby SAP binds directly to the phosphorylated ITSM domains of the SLAM receptors and then binds to the SH3 domain of Fyn through its SH2 domain, resulting in recruitment of the tyrosine kinase Fyn and initiation of signal transduction (122). SAP may also serve as docking site for the SH3 domain of protein kinase C-θ (PKC-θ), which has been shown to be important for SAP-dependent IL-4 production by CD4+ T cells (123).

Mutations in the Sh2d1b1 gene have been shown to lead to X-linked lymphoproliferative disease (XLP). This mutation results in three major disease features:
fulminant infectious mononucleosis, B cell lymphomas, and dys-gammaglobulinemia (103, 124). Studies from mouse knockout models have shown that while IL-4 production is impaired in SAP\(^{-/-}\) T cells, IFN-\(\gamma\) production is increased following stimulation with anti-CD3 and -CD28 mAbs. Consistent with these \textit{in vitro} findings, SAP knockout mice are hyper-responsive to lymphocytic choriomeningitis virus infection, with elevated levels of IFN-\(\gamma\)–producing cells in the spleen and liver (125, 126).

### 1.3.4 EAT-2

EAT-2 is homologous with SAP, with \(~50\%\) of amino acid sequence identity. It has been shown to bind to the tyrosine phosphorylated ITSM motifs of CD150, Ly9, CD84, 2B4, and CRACC following receptor engagement (54,56) (104, 127). Unlike SAP, EAT-2 cannot bind Fyn because it lacks the necessary arginine at position 78 in the carboxyl terminal (117, 128); instead, tyrosine residues located in the carboxyl terminal tail (1 in humans, 2 in mice) convey inhibitory signals when phosphorylated (120, 129).

The role of EAT-2 has been most extensively studied in NK cells. Examination of the function of NK cells in EAT-2 knockout mice demonstrated that EAT-2\(-/-\) NK cells secrete increased amounts of IFN-\(\gamma\) in response to various stimuli, including SLAMF4 (2B4), CD16, NK group 2, member D (NKG2D) and lymphocyte antigen 49D (Ly49D) activation. The ability of NK-cells to kill certain targets is also augmented in EAT-2 deficient mice (127). Similar findings were observed for ERT\(-/-\) NK cells, suggesting that ERT also acts as a negative regulator of NK cell function. The mechanism of NK-cell inhibition by EAT-2 and ERT remains to be fully clarified, but could involve binding of protein tyrosine phosphatases,
inhibitory kinases or ubiquitin ligases to the phosphorylated tails of EAT-2 and ERT. However, not all studies have shown an inhibitory role for EAT-2. In a study examining the role of EAT-2 downstream of SLAMF7 (CD319, CRACC) activation, NK cell-mediated cytotoxicity was impaired in EAT-2/- NK cells (130).

1.4 Dendritic cells

Dendritic cells (DCs) are antigen-presenting cells (APC) that play a key role in both innate and adaptive immunity, and provide antigen (Ag) to T cells to induce immune responses. As an initiator of adaptive immunity, immature DCs capture antigens derived from infectious pathogens, tissue necrosis, and local inflammation in peripheral tissues. In the presence of these 'danger' signals as well as pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, prostaglandins, and tumor necrosis factor (TNF)-α, DCs undergo a maturation and activation process (131, 132). Depending on the cytokine milieu, naïve T cells then proliferate and differentiate into Th1, Th2, Tfh (T follicular helper cells) or Treg cells with diverse cytokine production profiles: interferon (IFN)-γ (Th1), interleukin (IL)-4/IL-13 (Th2), IL-21 (Tfh) and IL-10/transforming growth factor (TGF)β (Treg), respectively (133), resulting in the induction of immunity or tolerance. Several reports show that immature (non-activated) DCs act as inducers of T cell tolerance in the periphery after capturing self-antigens (e.g. apoptotic cells) (134-136), whereas mature antigen-loaded DCs induce antigen-specific immunity (137, 138). Given the roles of DCs in the regulation of immune responses and tolerance, it is not surprising that abnormal DC activation has been shown to skew self-antigen presentation from tolerance to autoimmunity in SLE.
1.4.1 Myeloid and Plasmocytoid dendritic cell subsets

DCs constitute a complex system of cells, which comprise several subsets and display different characteristics and tissue distributions. Discussion of all the DC subsets is beyond the scope of this thesis; however, DCs can be classically divided into two distinct subsets: conventional dendritic cells (cDCs) and plasmacytoid DC (pDC). cDC can also be divided into a number of subsets according to their localization. Myeloid dendritic cells (mDC), a typical cDC subtype, can be differentiated from human monocytes or mouse bone marrow in vitro in the presence of GM-CSF, and are usually CD11c⁺CD11b⁺B220⁻ cells. pDCs can also be differentiated from bone marrow-derived cells or human monocytes but require the presence of Flt-3 ligand (Flt-3L) for their generation (reviewed at (139)).

1.4.1.1 Role of mDCs in the pathogenesis of lupus

Considerable evidence suggests that defective clearance of apoptotic cells by macrophages plays an important role in the pathogenesis of lupus (140, 141). mDCs have been shown to take up apoptotic and necrotic cell material and present it to T cells (137, 140). While uptake of early apoptotic cells promotes tolerance, uptake of inefficiently cleared later apoptotic or necrotic cells leads to mDC activation and activation of T cells. Indeed, many of the potential endogenous ligands that can activate DCs, including RNA, DNA, and HMGB1, are found in the apoptotic blebs released by late apoptotic cells. Uptake of this late apoptotic debris by immature mDCs induces their maturation, leading to upregulation of CD40 and costimulatory molecules as well as production of pro-inflammatory cytokines, such as IL-12 and IL-6 (Reviewed in (139, 142)). Presentation of autoantigens derived from apoptotic debris
by activated mDCs to autoreactive T cells is proposed to induce differentiation of Th1, Th17 and Tfh cells. In particular, production of IL-6, together with other pro-inflammatory cytokines, acts to promote differentiation of Th17 cells and Tfh cells, and inhibit differentiation/activation of Treg cells (143). Production of IL-6 by activated mDCs also promotes the survival and differentiation of B cells (144), which together with T cell help leads to the production of autoantibodies. Activated mDCs are also a source of the B-cell activating factor, BAFF, which further promotes B cell activation, survival, and autoantibody production (145).

In mice, mDCs can also take up early apoptotic antigens that are complexed with autoantibodies in an Fc-dependent manner (such as receptors for the Fc region of IgG (FcyR)), leading to activation of TLR7 and TLR9 by endogenous RNA and DNA in the apoptotic debris, respectively (146, 147). Thus, once autoantibodies are produced that can bind to the improperly cleared apoptotic debris, immune complexes further enhance the uptake of apoptotic debris by mDCs, resulting in further augmentation of pro-inflammatory cytokine production, contributing to a positive feed-back loop (148).

Several studies have revealed that functional abnormalities of mDCs promote the development of lupus (reviewed at (139)). For example, induced mutations or susceptibility alleles that lead to enhanced production of pro-inflammatory cytokines in response to TLR signaling, such as the Sigirr knockout or the yaa allele, promote lupus. There is evidence that DC function is also defective in the NZ mouse strains. The mDCs of B6.Sle1.Sle2.Sle3 (B6.TC) triple congenic mice, which have NZM2410-derived susceptibility loci from chromosomes 1, 4, and 7 backcrossed on a B6 background, accumulate in the bone marrow, spleen and lymph nodes as compared to B6 controls (149). Although bone marrow-derived myeloid DCs from these mice express lower levels of CD80, CD86, and MHC class II, they induce more
proliferation in CD4+ T cells and inhibit the suppressive activity of Treg cells, as compared to B6 DCs. In support of a role for IL-6 in the inhibition of Treg development in these mice, B6.TC DCs display enhanced production of IL-6, which was shown to lead to blockade of Treg activity. Interestingly, the increased production of IL-6 by DCs and blockade of Treg activity in B6.TC both mapped to the Sle1 locus. In further support of a role for IL-6 in the pathogenesis of disease in NZ mice, inhibition of IL-6 ameliorated kidney disease and reduced the titers of anti-dsDNA antibodies in the NZB/W model (150).

Although there is evidence that mDC function may also be altered in SLE patients, the data are somewhat contradictory. One group reported that monocyte-derived DCs (Mo-DCs) from SLE patients, which have similar features to mDCs, were decreased in number and had lower surface levels of HLA-DR, CD86 and CD80, as well as an impaired ability to stimulate T cells, compared to those from normal healthy controls (151). In contrast, others have reported higher levels of HLA-DR, CD86 and CD80, and an enhanced ability to stimulate allogeneic T cell proliferation responses in-vitro for Mo-DC from SLE patients (152).

1.4.1.2 Role of pDCs in the pathogenesis of lupus

pDCs are rare circulating innate immune cells (0.2-0.8% of leukocytes) that are potent producers of type I interferons (IFN-α/β) in response to viral nucleic acids sensed through TLR7 and TLR9 (153, 154). Type I IFNs are pivotal in the activation of the innate and adoptive immune system and in the antiviral response (155-158). In addition to virus-derived DNA and RNA, pDCs can also be activated by self-nucleic acids complexed with antibodies (159) or DNA/RNA-binding proteins such as HMGB1(160).
Several lines of evidence support an important role for type I IFNs in the pathogenesis of SLE (reviewed in (161, 162)). IFN-α overexpression strongly exacerbates experimental SLE and administration of therapeutic doses of IFN-α can lead to development of SLE in humans (163). Hence, the blockade of IFN signaling (for example, using antibodies to IFN or IFNAR(IFN-α receptor)) represents a potential therapeutic approach to SLE (164). Indeed, several groups have found a higher expression of IFN-responsive genes in peripheral blood cells of lupus patients, which is known as the “Interferon Signature” (165-167).

Lupus mouse models also support a role for type I IFNs in the initiation of autoimmunity in lupus. Deletion of the type I IFN receptor (IFNAR1) in NZB mice ameliorates disease activity resulting in reduced mortality, hemolytic anemia, and GN as well as diminished production of autoantibodies (168). Similar findings were reported in other lupus mouse models such as C57BL/lpr, (B6.Nba2 × NZW)F1 and NZM2328 when IFNAR1 was deleted in these mouse models (90, 169-171). In contrast, MRL/lpr mice deficient in IFNAR1 showed augmented disease (172), despite the observation that MRL/lpr have increased expression of type I IFN responsive genes (173). In addition, deletion of the IFNAR1 gene had a minimal impact on B6.Nba2 mice (174). These latter finding suggests that type I IFN may not play an important role in the exacerbation of all lupus-prone mouse models.

Type I IFNs have pleomorphic effects on the immune system, most of which are predicted to augment the immune dysregulation in lupus. IFN-α promotes the differentiation of monocytes to antigen-presenting cells (175) and induces the maturation and activation of mDCs (162), promoting increased differentiation and activation of T cells. IFN-α also promotes the polarization of T cells to Th1 cells, suppresses Treg cell differentiation, and directly activates
cytotoxic T cells (176). Furthermore, IFN-α activates B cells to differentiate into antibody-producing plasma cells (177) and enhances the cytotoxicity of NK cells (178).

As pDCs produce large amounts of type I IFNs, it has been proposed that they are the major source of pathogenic IFN in SLE. In support of this, IFN-producing pDCs have been found infiltrating various inflamed tissues, such as the skin and kidneys, of SLE patients. Several studies in humans and mice have shown that immune complexes (IC) containing nucleic acids, including dsDNA and RNA, induce IFN-α production by pDCs (reviewed in (179, 180)). Uptake of these immune complexes is mediated by receptors for the Fc region of IgG (FcγR), in particular, FcγRIIa (159), which targets the complex to the late endosomal compartment, where the nuclear antigens within the complex can bind to and stimulate relevant TLRs (TLR7 for RNA and TLR9 for dsDNA). Although free dsDNA and RNA is not taken up by pDCs, recent work suggests that dsDNA complexed with the anti-microbial proteins found in neutrophil extracellular traps can also be taken up in a FcγR-independent fashion, leading to TLR stimulation. In addition to being the major producers of type I IFNs, pDCs can process and present autoantigen to autoreactive T cells, further promoting their activation and proliferation.

Although pDC function appears to be mostly normal in SLE, there is some evidence that pDCs may be functionally abnormal in NZ mouse strains. The pDCs from NZB mice have been shown to secrete high levels of IFN-α when stimulated with a TLR9 agonist in-vitro (181). Subsequent studies in B6.Nba2 subcongenic mice have linked this abnormality to an interval containing both the FcγR and SLAM loci (91) and revealed that this interval controls expansion and TLR9 sensitivity of pDCs, leading to enhanced production of cytokines such as IL-10 and IFN-α. Indeed, work from our laboratory also showed marked expansion of splenic pDC and
mDC populations in B6.NZBc1c13 biconegenic mice that contain both c1 (extending from 62-191 Mb) and c13 (extending from 37.7-119.6 Mb) intervals (182). However, despite expansion of pDCs in B6.NZBc1c13 biconegenic mice, splenic IFN-α levels remained low and production of IFN-α by pDCs was reduced in older mice. Previous experiments in our laboratory indicate that B6.NZBc13 mice have a defect in clearance of apoptotic debris by macrophages, resulting in increased availability of TLR-stimulating nuclear antigens (183), so it was hypothesized that chronic exposure to nuclear antigen-containing ICs in vivo might lead to impaired IFN-α production. This hypothesis was examined by repeated stimulation of bone marrow pDCs with TLR ligands, and the results showed that chronic stimulation of pDCs with TLR ligands leads to impaired IFN-α production, a phenomenon termed TLR tolerance. These observations suggested that expansion of pDCs and production of anti-nuclear antibodies is not necessarily associated with increased IFN-α production. Nevertheless, B6.NZBc1c13 biconegenic mice were found to produce high levels of other pro-inflammatory factors such as IFN-γ, TNF-α and BAFF, raising the possibility that the expansion of DCs in these mice may arise from increased amounts of pro-inflammatory factors rather than TLR-hyper-responsiveness. Abnormal production of pro-inflammatory cytokines was linked to the genetic loci on chromosome 1, as increased levels of these cytokines, with the possible exception of TNF-α, were not seen in B6.NZBc13 mice.

Expansion of pDCs also has been reported in B6.TC mice. However, mDCs from B6.TC mice expressed a type I IFN signature before initiation of disease, suggesting that mDCs may be more relevant before disease onset (149, 169, 184). In support of a role for both mDCs and pDCs in the pathogenesis of lupus, it has been demonstrated that depletion of mDCs and pDCs in the MRL/lpr model ameliorated the lupus phenotype (185).
1.5 Role of T cells in the pathogenesis of SLE

CD4+ T helper (Th) cells are active mediators of SLE pathogenesis, and have been shown to play an important role in disease pathogenesis in a number of induced mutant and spontaneously arising mouse models of lupus as well as human lupus (186-188).

The contribution of different effector T-helper cell subsets to the pathogenesis of lupus has been the subject of much research over the years. Several studies have been conducted in different lupus mouse models, indicating the central role of Th1, Th17 and Tfh cells in the pathogenesis of lupus. Earlier studies had indicated a predominant role for Th1 cells in lupus (189, 190), however increasing data suggest an important role for T follicular helper cells (Tfh) and Th17 cells in lupus (191). However, some disagreement still exists regarding the relative contributions of specific Th cell subsets and their related cytokines to the pathogenesis of lupus. Evidence demonstrating the role of different T cell subsets in the pathogenesis of lupus will be discussed in the following sections.

1.5.1 Role of Th1 cells in the pathogenesis of lupus

Th1 self-reactive T cells have been identified in several murine models of lupus and are thought to provide support for autoantibody production (192-195). The first evidence showing the importance of IFN-γ signaling in the pathogenesis of murine lupus was provided by Ozmen et al., who showed that blockade of IFN-γ with antibodies against IFN-γ or the IFN-γ receptor (IFN-γR) resulted in inhibition of kidney disease in NZB/W mice (193). Subsequent reports confirmed this observation by showing that genetic disruption of the Ifngr gene in MRL/lpr and NZB/W lupus-prone mice led to reduced autoantibody production and kidney damage (194-
Similar findings were observed when IFN-γ was blocked by treatment with IFN-γR-Fc (197) or by knock out of the T-box transcription factor (T-bet) that plays a critical role in Th1 lineage commitment, where decreases in serum levels of pathogenic autoantibodies such as IgG2a and IgG3 paralleled reduction in kidney disease (198). Th1 cells and their main effector cytokine, IFN-γ, have also been implicated in the development of lupus in humans (199). Several studies have shown increased proportions of Th1 cells in the peripheral blood of SLE patients compared to healthy individuals (200).

Despite the evidence strongly implicating IFN-γ in the pathogenesis of lupus, the precise pathogenic mechanism by which this cytokine contributes to disease is poorly understood. Previous reports suggested that IFN-γ contributes to the development of lupus through its capacity to induce B cell immunoglobulin (Ig) switching to IgG2a and IgG3 isotypes that are particularly efficient at activating downstream inflammatory cascades (201, 202). However, a recent report demonstrating that T cell-derived IFN-γ does not directly stimulate antibody production by human B cells (203) suggests that B cells may not be the direct target of IFN-γ in its pathogenic role to induce the disease (203).

Indirect effects of IFN-γ on B cells could be mediated via stimulation of myeloid cells to produce BAFF, thereby supporting aberrant B cell responses (204, 205). Interestingly a recent report demonstrated a fundamentally different mechanism of action of IFN-γ. They showed that IFN-γ induced accumulation of Tfh cells, resulting in abnormal GC formation and autoantibody production in the sanroque mouse model of lupus (206).
1.5.2 Role of Tfh cells in the pathogenesis of lupus

Tfh cells are a distinct subset of Th cells involved in the regulation of antigen-specific B cell immunity by, providing signals and cytokines required for clonal expansion, class switching, plasma cell differentiation and formation of germinal centers (207, 208). Tfh cells express the unique transcription factor Bcl6 (209, 210) and are characterized by expression of high levels of CD40L, ICOS, PD1 and most importantly, CXCR5, which allows them to localize to developing germinal centers (211-214). These cells characteristically produce high levels of IL-21, a cytokine that has been shown to be critical in the generation of plasma cells, isotype switching, and germinal center formation (211, 215).

The role of this population in lupus was first suggested when investigations into the sanroque mouse strain revealed that a point mutation in the gene encoding Roquin was associated with an intrinsic T cell defect that resulted in spontaneous expansion of Tfh cells and increased germinal center formation (216). These mice developed a lupus-like disease, characterized by ANA production and GN. Notably, introduction of a deletion in SLAM-associated protein (SAP) onto this background, which inhibits Tfh cell development, resulted in correction of autoimmunity and normalization of the number of Tfh cells (217). This finding raised the possibility that abnormal help provided by deregulated Tfh cells might promote lupus. In support of this concept, increased proportions of Tfh cells that produce high levels of IL-21 have also been seen in MRL/lpr and BXSB/Yaa lupus-prone mice (218, 219). Deletion of IL-21 receptors in BXSB/Yaa mice was associated with decreased numbers of Tfh cells and ameliorated development of pathogenic autoantibody production, GN and mortality (218). Increased numbers of circulating Tfh-like cells have also been observed in human SLE patients, and are associated with more severe disease (203, 220). Additionally, IL-21
polymorphisms are associated with human SLE (221). Taken together, the existing evidence suggests that this cell population may play a pathogenic role in both human and murine lupus.

1.5.3 Role of Th17 cells in the pathogenesis of lupus

Th17 cells have been shown to develop via a pathway distinct from other Th lineages as they uniquely express orphan retinoid nuclear receptor (ROR)γt for their differentiation (222), which acts synergistically with RORα to induce complete Th17 differentiation (223). Lineage commitment to Th17 cells from naïve T cells is induced by the combination of transforming growth factor (TGF)-β and IL-6 cytokines, or IL-21 stimulation (224-226). Th17 cells produce IL-17A and IL-17F, which are potent pro-inflammatory cytokines involved in the early immune response against extracellular bacteria and fungi (29). They also produce IL-21, IL-22, TNF, IL-6 and IL-9 (227-229).

Studies in lupus-prone mice provide evidence for the participation of Th17 cells in lupus pathogenesis. Splenocytes from (NZB x SWR)F1 mice produce significant levels of IL-17 when stimulated with nucleosomes and Th17 cells are also found in the kidneys of these mice (230). In NZM2328 mice with a gene deletion of TNF receptors 1 and 2, increased numbers of Th17 cells were seen and associated with high anti-dsDNA antibody levels and accelerated nephritis (231). Increased serum levels 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 (232). BXD2 lupus-prone mice exhibited increased production of IgG anti-DNA antibodies, as well as spontaneous development of elevated numbers of germinal centers. Introduction of a null gene for the IL-17A receptor onto the
BXD2 background drastically reduced the number of germinal centers in the spleen and ameliorated production of IgG autoantibodies and nephritis, indicating that IL-17 plays a pathogenic role in these mice (233). Increased proportions of IL-17 producing cells, which infiltrate the inflamed kidneys, skin, and lungs, are also seen in human SLE patients, suggesting a similar pathogenic role in humans (233-236).

Thus, increases in Th1, Th17 and Tfh cell subsets appear to be associated with a severe lupus phenotype, in particular renal disease. The proposed mechanisms by which Th1, Tfh and Th17 cells drive SLE autoimmunity are summarized in Figure 4. Currently, the precise immune mechanisms leading to the increased generation of these cells in lupus are yet to be identified.
**Figure 1.4. Proposed model on Th1, Tfh and Th17 cells contribution to SLE pathogenesis.** Th1 cells produce large amounts of IFN-γ, inducing accumulation of Tfh cells and promoting differentiation of autoreactive B cell to plasma cells that produce pathogenic IgG2a and IgG3 isotypes. In parallel, production of IL-21 by Tfh cells promotes survival, class-switching, and production of antinuclear autoantibodies by autoreactive B cells and Th17 cells differentiation. Th17 cells also produce IL-21, providing an autocrine signal, which is important for effective Th17 differentiation. Ultimately, Th17 cells infiltrate the kidneys, and through production of IL-17 activate macrophages, renal fibroblasts and epithelial cells to produce pro-inflammatory chemokines and cytokines. In addition, antibody production by autoreactive B cells can complex with nuclear Ag and complement factors, activating macrophages, and in tandem with IL-17 this leads to production of cytokines and chemokines such as G-CSF, GM-CSF, IL-6, TNFα, CXCL1, CXCL2 and CXCL5. This results in the recruitment and activation of neutrophils, which together with pro-inflammatory cytokines and complement activation, leads to kidney damage.
1.6 Thesis objectives and hypothesis

Experiments in the Wither laboratory have focused on the NZB mouse strain, one of the first lupus-prone murine models discovered. This strain spontaneously develops an autoimmune disease that is remarkably similar to human SLE.

Our laboratory has identified a region on NZB c1 that is linked to the development of GN and production of anti-nuclear antibodies. B6 congenic mice with a NZB c1 interval, extending from 35-106 cM, produce anti-nuclear antibodies and develop mild GN. By creating mice with smaller overlapping NZB c1 intervals, in previous work we have demonstrated that there are at least three lupus susceptibility loci located on NZB c1 within the 70cM to 100cM region, that are sufficient to induce disease. In these preliminary studies, T cells from young pre-autoimmune mice (which were predominantly naïve) demonstrated increased production of IFN-γ in response to anti-CD3 stimulation, suggesting the presence of an intrinsically altered T cell function. This was further supported by results in mixed hematopoietic chimeric mice, where B6.NZBc1 demonstrated enhanced spontaneous T cell activation compared to their B6 counterparts. Thus, one or more of the loci appeared to promote lupus by altered T cell activation and/or differentiation.

The overall objective of my studies was to identify the lupus susceptibility genes within the c1(70-100) interval and to define the immune mechanisms by which they act to promote disease with the following hypothesis:

Intrinsic T cell defects in c1(70-100) mice lead to increased differentiation to, and/or altered function of, Tfh and/or Th17 cells, and the differences in the severity of renal disease
in subcongenic mouse strains with shorter regions in this interval reflect differences in their capacity to promote differentiation of these T cell subsets.

Therefore the initial Aims of my thesis were:

**Aim 1.** To characterize the pro-inflammatory T cell subsets in c1(70-100) mice and to localize the genetic regions associated with the observed abnormalities using c1 subcongenic mouse strains.

**Aim 2.** To determine whether alterations in the proportions of various T cell populations in NZB c1 congenic mouse strains arise from intrinsic T cell functional abnormalities and/or altered function in other cell populations.

Findings from Aims 1 and 2, outlined in chapter II of the thesis, suggested that genetic polymorphisms on NZB c1, located in the 70-100 cM interval, resulted in intrinsic T cell and other immune cell defects, one of which was localized to DC, that interacted in an epistatic fashion to enhance differentiation of autoreactive and antigen-primed T cells to IL-17-, IL-21- and IFN-γ-producing cells, promoting renal disease in these mice.

Since these findings localized the genetic polymorphism leading to altered DC function to the 88-96 interval of NZB c1 congenic mice, which contains only 18 protein coding genes, my subsequent experiments focused upon identification of the candidate gene within this interval. These experiments are outlined in Chapter 3 of thesis and had the following specific aim:

**Aim 3.** To identify the candidate gene in the NZB 88-96 interval and determine how it leads to the altered DC function in these mice.
Chapter 2

T cell and Dendritic cell Abnormalities Synergize to Expand Pro-inflammatory T cell Subsets Leading to Fatal Autoimmunity in B6.NZBc1 Lupus-Prone Mice

Nafiseh Talaei1,2, Yui-Ho Cheung1,2, Carolina Landolt-Marticorena3,4, Babak Noamani1, Timothy Li1 and Joan E. Wither1,2,3,4

1Arthritis Centre of Excellence, Division of Genetics and Development, Toronto Western Research Institute, University Health Network, Toronto, Ontario; Departments of 2Immunology and 3Medicine, University of Toronto, Toronto, Ontario; 4Division of Rheumatology, University Health Network, Toronto, Ontario

All experiments were performed by N. Talaei. Y. Cheung contributed to generation of c1 congenic mice. C. Landolt and B. Noamani helped with sacrificing of mice and isolation of organs, T. Li performed the genotyping.

2.1 Abstract

We have previously shown that B6 congenic mice with a New Zealand Black chromosome 1 (c1) 96-100 cM interval produce anti-nuclear Abs and that at least two additional genetic loci are required to convert this subclinical disease to fatal glomerulonephritis in mice with a c1 70-100 cM interval (c1(70-100)). Here we show that the number of T follicular helper and IL-21-, IFN-γ-, and IL-17-secreting CD4+ T cells parallels disease severity and the number of susceptibility loci in these mice. Immunization of pre-autoimmune mice with OVA recapitulated these differences. Differentiation of naïve T cells in-vitro under polarizing conditions and in-vivo following adoptive transfer of OVA-specific TCR transgenic cells into c1(70-100) or B6 recipient mice, revealed T cell functional defects leading to increased differentiation of IFN-γ- and IL-17-producing cells in the 96-100 cM and 88-96 cM intervals, respectively. However, in-vivo enhanced differentiation of pro-inflammatory T cell subsets was predominantly restricted to c1(70-100) recipient mice, which demonstrated altered dendritic cell function, with increased production of IL-6 and IL-12. The data provide support for the role of pro-inflammatory T cells in the conversion of subclinical disease to fatal autoimmunity and highlight the importance of synergistic interactions between individual susceptibility loci in this process.
2.2 Introduction

Systemic Lupus Erythematosus (SLE) is a generalized autoimmune disease characterized by the production of autoantibodies, particularly those directed against nuclear antigens, which form immune complexes that deposit in tissues. Studies of SLE in humans and lupus-prone mice indicate that multiple genetic polymorphisms affecting diverse immune populations interact with each other to produce the lupus phenotype. Among these populations are T helper (Th) cells. Although early studies demonstrated a predominant role for Th1 cells in lupus, several recent studies suggest that two other pro-inflammatory Th cell subsets, T follicular helper (Tfh) and Th17 cells, are also pathogenic (237).

Tfh cells are a distinct subset of Th cells that provide help for antigen specific B cell responses in the context of germinal centers (GC) and produce high levels of IL-21 (207, 208). A potential role for this population in the pathogenesis of lupus was first suggested by the observation that lupus-prone mice with a homozygous point mutation in the Roquin gene, demonstrated expansion of their Tfh population, and subsequently supported by demonstration of similar expansions in MRL<sup>lpr</sup> and BXSB/Yaa lupus-prone mice (238).

Although Th17 cells are defined by their IL-17 production, they produce a variety of other cytokines including IL-21, IL-22, TNF-α, IL-6 and IL-9 (239). Expansion of this population has been demonstrated in several lupus-prone mouse strains, including (New Zealand Black (NZB) x SWR)<sub>F1</sub>, TNF receptor 1 and 2 gene-deleted New Zealand Mixed 2328, and BXD2 mice (230-232). Notably, introduction of a null gene for the IL-17A receptor onto the BXD2 background significantly attenuated production of IgG autoantibodies and nephritis (232). Despite compelling evidence that Tfh and Th17 cells play a central role in lupus
pathogenesis, the genetic basis leading to the aberrant activation of these cell populations remains unknown.

To characterize the immunologic abnormalities that promote lupus, our laboratory has produced a series of congenic mouse strains with homozygous NZB chromosomal intervals crossed onto the non-autoimmune C57BL/6 (B6) background. In previous experiments we showed that mice with a NZB c1 interval extending from 70-100 cM (c1(70-100)) develop a severe lupus phenotype, with high titers of anti-dsDNA Abs and glomerulonephritis (GN), leading to death of ~40% of the mice by 8 months of age. This phenotype appeared to result from at least 3 genetic loci, as indicated by progressively attenuated disease in mice with NZB c1 intervals extending from 88- or 96-100 cM (94). Here we show that the disease severity in these mice parallels the expansion of pro-inflammatory T cell subsets, specifically Th1, Th17, and Tfh cells. We further demonstrate that this expansion can be recapitulated following immunization of pre-autoimmune mice with an exogenous antigen. This T cell skewing results from a combination of immune cell functional abnormalities in congenic mice that localize to different regions within the c1 70-100 interval. Naïve T cell functional abnormalities that lead to expansion of IFN-γ- and IL-17- producing cells localized to the 96-100 and 88-96 intervals, respectively, whereas DC functional abnormalities that promote expansion of all the pro-inflammatory T cell subsets localized to the 88-96 and 70-88 intervals. Notably, altered DC function appeared to play a critical role in this expansion, because in the absence of DC abnormalities, minimal expansion of pro-inflammatory T cell subsets was seen. Our findings provide insight into how individual susceptibility loci, which alone produce modest changes in immune function, interact synergistically to profoundly alter immune function leading to severe clinically relevant autoimmune disease.
2.3 Materials and Methods

2.3.1 Ethics statement

Mice were housed in a Canadian Council on Animal Care approved facility at the Toronto Western Research Institute, part of the University Health Network. All mice used and experiments performed in this study were approved by the Animal Care Committee of the University Health Network (Animal Use Protocol #123).

2.3.2 Mice

B6 and NZB mice were purchased from Taconic (Germantown, NY) and Harlan Sprague Dawley (Blackthorn, England), respectively. B6.OT-II TCR Tg and B6.Thy1aIgHa mice were originally obtained from Taconic and The Jackson Laboratory (Bar Harbor, ME), respectively, and bred in our facility. Congenic mice were generated as previously described (94). OT-II TCR Tg and Thy1aIgHa (termed Thy1.1 for simplicity) congenic mice were produced by polymorphic marker assisted backcrossing. Only female mice were examined and all mice were specific-pathogen free.

2.3.3 Flow cytometry

Half a million RBC-depleted splenocytes were incubated with mouse IgG (Sigma-Aldrich) for 15 min prior to staining with various combinations of directly-conjugated mAbs. Allophycocyanin- or PerCP-Cy5.5-conjugated streptavidin (SA) (BD Biosciences) were used to reveal biotin-conjugated Ab staining. Dead cells were excluded by staining with 0.6 μg/ml PI (Sigma-Aldrich). Events were acquired using a LSR II instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc.; Ashland, OR). The following directly
conjugated mAbs were purchased from BD Biosciences: Biotin-conjugated anti-CXCR5 (2G8), -CD3 (145-2C11), -CD4 (RM4-5), -B220(RA3-6B2), -CD86 (B7.2; GL1), and -CD90.2 (Thy1.2; 30-H12); PE-conjugated anti-CD69 (H1.2F3), -CD44 (IM7), -CD95 (Jo2), -B7.2 (GL1), -IA/IE (M5/114.15.2) and -PD1 (J43); PE-Cy7 conjugated anti-CD44 (IM7) and -CXCR5 (2G8); Pacific Blue-conjugated anti-B220 (RA3-6B2) and -CD4 (RM4-5); PerCP-Cy5.5-conjugated anti-B220 (RA3-6B2); and FITC-conjugated anti-CD90.1 (Thy1.1; OX-7) and -CD11b (M1/70). All isotype controls were obtained from BD Biosciences. Biotin-conjugated peanut agglutinin (PNA) was purchased from Sigma-Aldrich and FITC-conjugated anti-CD62L (MEL-14) mAb was purchased from Cedarlane Laboratories (Burlington, ON, Canada).

2.3.4 Detection of cytokine-secreting T cells

CD4+ T cells were isolated from RBC-depleted splenocytes using the Dynal Mouse CD4 Negative Isolation Kit (114.15D, Invitrogen), re-suspended in complete RPMI medium (10% FBS, non-essential amino acids, L-glutamine, β-mercaptoethanol, penicillin, and streptomycin), and stimulated at 2.5 x 10^5 cells per well in 96-well plates with plate-bound anti-CD3 Ab (4μg/ml; Cedarlane) and 1 μg/ml soluble anti-CD28 Ab (BD Biosciences). Supernatants were harvested after 72 h and the levels of IL-2, IL-4, IL-17, and IFN-γ measured using a mouse cytometric bead array kit specific for Th1/Th2/Th17 cytokines (BD Biosciences). IL-21 levels were measured using a mouse IL-21 Duo-Set ELISA kit (R&D Systems). Cytokine-secreting CD4+ T cells were detected by flow cytometry. RBC-depleted splenocytes were stimulated for 5 h with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences). The cells were stained with Pacific Blue-anti-CD4
and biotinylated-anti-CD3 followed by PerCP-Cy5.5-SA, and then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) before intracellular staining with allophycocyanin-anti–IFN-γ (XMG1.2), Alexa Fluor® 488-anti-IL-17A (TC11-18H10), and PE-anti–IL-4 (BVD4-1D11). To quantify IL-21-secreting cells, fixed and permeabilized cells were incubated with an IL-21R/Fc chimera (R&D Systems) and then stained with a PE-conjugated affinity-purified F(ab’)2 goat anti-human Fc Ab (Jackson ImmunoResearch Laboratories) (240).

2.3.5 Naïve CD4⁺ T cell isolation and differentiation

Naïve CD4⁺ T cells (CD4⁺CD62L⁺) were purified using a mouse CD4⁺CD62L⁺ T Cell Isolation Kit (Miltenyi Biotec), re-suspended in complete RPMI, and stimulated with plate-bound anti-CD3 (4 μg/ml) and soluble anti-CD28 (1 μg/ml) in 96-well plates under the following conditions (all cytokines and mAb purchased from R&D Systems): Th0 cell: anti-IFN-γ (10 μg/ml) and anti-IL-4 (10 μg/ml); Th1 cell: IL-12 (10 ng/ml), and anti-IL-4 (10 μg/ml); Th2 cell: IL-4 (10 ng/ml), and anti-IFN-γ (10 μg/ml); Th17 cell: IL-6 (10 ng/ml), IL-23 (10 ng/ml), TGF-β1 (2.5 ng/ml), anti-IFN-γ (10 μg/ml) and anti-IL-4 (10 μg/ml); and IL-21-producing T cell: IL-6 (30 ng/ml), anti-IFN-γ (10 μg/ml) and anti-IL-4 (10 μg/ml). After 4 days, the cells were washed and re-stimulated for 4 h with 50 ng/ml PMA and 1 μg/ml ionomycin in the presence of GolgiStop. Cytokine-secreting T cells were quantified as outlined previously.
2.3.6 In-vivo differentiation of OVA-specific T cells

Mice (8-12-wk old) were immunized i.p. with 100 μg OVA (Grade II, Sigma) or PBS emulsified in CFA (Sigma) and sacrificed 2 wks later. For measurement of OVA-specific cytokine production, 1 x 10^6 RBC-depleted splenocytes were cultured in complete RPMI (5% FBS) alone, or containing 100 μg/ml OVA, per well in 96-well plates. Supernatants were harvested at 72 h and assayed for IL-4, IFN-γ, IL-17, and IL-21, as outlined previously. For adoptive transfers, 3 x 10^6 naive splenic CD4+ T cells from 8-10-wk-old B6 or congenic OT-II mice were injected into the tail vein of 8-10-wk-old B6.Thy1.1 or c1(70-100).Thy1.1 recipients. The following day mice were immunized with OVA emulsified in CFA. The proportion of various T cell subsets within the spleen was determined 2 wks later by flow cytometry after gating Thy1.2+ (transferred) T cells.

2.3.7 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% FBS/PBS. Sections were stained with biotinylated-PNA, allophycocyanin-conjugated anti-CD4, PE-conjugated anti-PD1 and FITC-conjugated anti-IgM F(ab’)2 (Jackson Immunoresearch), to detect Tfh cells within GC. Biotin staining was revealed using 7-amino-4-methylcoumarin-3-acetic acid-conjugated streptavidin as a secondary reagent (Jackson Immunoresearch). Stained sections were mounted with Fluoro-Gel (Electron Microscopy Sciences) and tissue fluorescence was visualized using a Zeiss Axioplan 2 imaging microscope (Oberkochen, Germany). Digital images were obtained using the manufacturer’s imaging system.
2.3.8 BMDC isolation and stimulation

Bone marrow cells were isolated by flushing femurs of 8–12 wk-old mice. After RBC lysis, the cells were re-suspended at $10^6$ cells/mL and cultured for 7 days with recombinant human FLT3L (20 ng/mL; R&D Systems) in complete RPMI. For TLR stimulation, $4 \times 10^5$ cells were cultured in 96-well flat-bottom plates for 24 h with media alone or containing various TLR ligands including: Imiquimod (2 µM), Poly (I:C) (50 µg/mL), CpG ODN 2216 or control (250 nM), CpG ODN 1826 or control (250 nM) (all from InvivoGen; San Diego, CA), or LPS (25 µg/mL; Sigma-Aldrich), as a positive control. The cells were then harvested and stained with anti-CD11c, -CD11b, and -B220. Staining with anti-CD86 (B7.2) and -MHC-II was used to assess cellular activation and intra-cellular levels of IL-12, IL-6 and TNF-α were used to assess cytokine production. GolgiStop or GolgiPlug (BD Biosciences) were added to cell cultures for the last 4 h of the incubation, prior to measurement of intracellular cytokines, which were detected using allophycocyanin-conjugated anti-IL-12 (C15.6), Alexa Fluor® 488-conjugated anti-IL-6 (MP5-20F3) and PE-conjugated anti–TNF-α (TN3-19.12). BD Horizon fixable viability stain 450 (FVS450) was used to exclude dead cells. To assess IFN-α and IL-23 production, cytokine levels in tissue culture supernatants were measured by ELISA kits as follows: IFN-α (PBL Biomedical Laboratories; Piscataway, NJ); and IL-23 (IL-23 Duo-Set, R&D Systems).

2.3.9 In-vitro culture of BMDCs and OVA-specific T cells

$2 \times 10^4$ BMDC were co-cultured with OVA 323-339 peptide (GenScript, Piscataway, NJ) and $2 \times 10^5$ naïve CD4+ T cells, isolated from the spleens of 8-10-wk-old B6.OT-II or c1 congenic OT.II mice, in the presence of 5 ng/ml recombinant mouse GM-CSF (R&D Systems).
for 4 days. Cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) in the presence of GolgiPlug or GolgiStop (BD Biosciences) for 4 h before harvesting. The cells were then stained for cell surface DC (CD11c, CD11b, B220) or T cell (CD3, CD4) markers, fixed, permeabilized, and stained for detection of intracellular cytokines, including IL-6, IL-12, IL-21, IL-17 and IFN-γ, as outlined previously.

2.3.10 In-vitro culture of splenocytes and OVA-specific T cells

Splenocytes were isolated from 5-6-wk-old B6.Thy1.1 or c1(70-100).Thy1.1 mice. Total splenocytes were seeded in 96-well U-bottom plates at 2x10^5 cells per well, then co-cultured for 72 hr with 1 μg/ml OVA 323-339 peptide (GenScript, Piscataway, NJ) and 2x10^5 purified naïve CD4+ T cells isolated from the spleens of 8-10-wk-old B6.OT-II or c1(70-100) congenic OT.II mice. PMA (50 ng/ml) and ionomycin (1 μg/ml) together with GolgiPlug or GolgiStop (BD Biosciences) were added for the last 4 h before harvesting. The cells were then stained for cell surface DC (CD11c, CD11b, B220), B cells (CD19 and B220) or T cell (CD3, CD4) markers, fixed, permeabilized, and stained for detection of intracellular cytokines, as outlined previously.

2.3.11 Statistical analysis

Comparisons of differences between groups of mice for continuous data were performed using one-way ANOVA followed by Dunns’ post-test for multiple comparisons. All statistical analyses were performed using GraphPad software (La Jolla, CA, USA).
2.4 Results

2.4.1 Expansion of pro-inflammatory CD4+ T cell subsets in NZB c1 congenic mice

B6 congenic mice with NZB c1 intervals extending from 96-100 cM (172.8-183.0 Mb; c1(96-100)), 88-100 cM (170.3-183.0 Mb; c1(88-100)) or 70-100 cM (126.6-183.0 Mb; c1(70-100)) demonstrate progressively more severe disease with increasing length of the c1 interval (Figure 2.1). Since increases in the number and size of GC paralleled disease severity in these mice, we postulated that changes in Th cell number/function were producing these differences. To address this possibility, Th cell subsets were examined in 4-mo-old B6 and congenic mice, using flow cytometry. As shown in Figure 2A&B, the proportion and number of Tfh cells (gated as CD4+CD44hiCD62LloCXCR5hiPD1hi) was significantly increased in c1(88-100) and c1(70-100) mice, whereas the level of these cells in c1(96-100) mice was similar to B6 mice. Consistent with the increases in Tfh, and our previous findings, there was a trend to increased proportions of GC B cells in all three congenic mouse strains with the greatest increase seen in c1(70-100) mice (Figure 2.2 A&B). The expansion of Tfh was further confirmed by immunofluorescence microscopy where increased numbers of Tfh cells were seen in the GC of c1(88-100) and c1(70-100) mice (Figure 2.2 C&D).
Figure 2.1. Genetic map of the c1 congenic mouse strains studied. 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 2007 (m37 release) mouse genome assembly (www.ensembl.org). Potential candidate genes within the interval are indicated above the chromosomal map. Phenotypic features of NZB c1 congenic mouse strains are shown to the right of the c1 congenic mice genetic map (94).
Figure 2.2. c1 congenic mice have an increased proportion of GC B and Tfh cells. Freshly isolated splenocytes from 4-mo-old B6, c1(96-100), c1(88-100), and c1(70-100) mice were stained with anti-B220 in combination with anti-Fas and PNA to assess the proportion of splenic GC B cells (B220^+^Fas^+^PNA^{hi}). (A) Shown are contour plots gated on PI-excluding splenocytes from B6 and c1(70-100) mice. Boxes indicate the regions that were used to define GC B cells, with the numbers above them indicating the proportion of cells in the gated population. (B) Scatterplot showing the proportions of GC B cells in the various mouse strains. Each point represents the determination from an individual mouse. Horizontal lines indicate the mean of each group examined. (C) Splenic sections from 4 month old B6, c1(96-100), c1(88-100) and c1(70-100) mice were stained with FITC anti-IgM (Green), biotinylated PNA followed...
by 7-amino-4-methylcoumarin-3-acetic acid-conjugated streptavidin (Blue), PE anti-PD1 (Yellow) and allophycocyanin anti-CD4 (Purple). Arrows indicate the location of Tfh cells within the germinal center for each mouse strain. Note the increased numbers of Tfh cells (white dots) distributed throughout the large germinal center in c1(70-100) and to a lesser extent c1(88-100) mice. Magnification= ×10. The scale bar indicates 100 µm. (D) Scatter plot showing the number of Tfh cells within GC. Each point represents the average number of Tfh cells per GC for an individual mouse, with 5-7 GC being counted per mouse. Horizontal lines indicate the mean of each group examined. Significance levels were determined by one-way ANOVA with Dunns’ post-test. The p values for significant differences between B6 and congenic mouse strains are shown with *p<0.05, **p<0.01, ***p<0.001. Bars with p values above denote significant differences between congenic strains.
To examine the other Th subsets, cytokine-producing CD4\(^+\) T cells were quantified by flow cytometry following stimulation of freshly isolated splenocytes for 4 hrs with PMA and ionomycin, and intracellular staining for IL-4, IFN-\(\gamma\), and IL-17 (Figure 2.3 C). There were no differences between strains in the proportion of IL-4 producing cells, but a trend to a progressive increase in IFN-\(\gamma\) and IL-17 producing T cells with increasing size of the NZB c1 interval was seen (Figure 2.3 D). Similar findings were obtained when CD4\(^+\) T cells were stimulated \textit{in-vitro} with anti-CD3 and -CD28 Abs, and secretion of various cytokines was quantified in the supernatants (Figure 2.4). While there was no significant difference between the mouse strains in the production of IL-2 and IL-4, there was a progressive increase in the secretion of IFN-\(\gamma\), IL-17, and IL-21 that correlated with increasing length of the c1 interval.

To further define the CD4\(^+\) T cell populations secreting these cytokines, intra-cellular cytokine levels were examined in cells stained with anti-CD3, -CD4, -CXCR5, and -PD1 to permit discrimination between Tfh (CD4\(^+\)CXCR5\text{hi}PD1\text{hi}) and conventional CD4\(^+\) T cells (including Th17 and extrafollicular T cells). This revealed that the increase in IL-21 and IFN-\(\gamma\) secreting cells observed in c1 mice results from increases in the numbers of both Tfh and conventional CD4\(^+\) T cells that secrete these cytokines (Figures 2.5 A&B), which positively correlated with the number of NZB genetic loci. A significant proportion of the IL-21 secreting cells also secreted IFN-\(\gamma\) (20-40% Tfh, 40-60% conventional), and conversely IFN-\(\gamma\) secreting cells also secreted IL-21 (40-60% Tfh, 30-50% conventional), with the proportion of co-secretors paralleling the length of NZB interval in congenic mice (Figure 2.5A).

The majority of IL-17 secreting cells were seen in the conventional CD4\(^+\) T cell population (Figure 2.5 B), with ~5% of the cells also secreting IFN-\(\gamma\) and 80% of cells secreting IL-21 in all mouse strains examined (Figure 2.5A). Consistent with the flow cytometry
findings, the majority of IL-17 secreting cells were seen within the T cell zone and the number of these cells was increased in c1(88-100) and c1(70-100) mice (Figure 2.5 C&D).
Figure 2.3. Expansion of Tfh, Th17 and Th1 cell subsets in c1 congenic mice.
Splenocytes from 4-mo-old mice were stained to assess the proportion of Tfh (CD4⁺CD44⁺CD62L⁻CXCR5⁺PD1⁺) cells. (A) Representative contour plots from B6 and c1(70-100) mice. Thick boxes denote the regions that were used to identify Tfh cells. Cells shown in the right panels were gated on the regions shown in the left panels. (B) Scatter plots showing the proportion of Tfh cells within the CD4⁺ T cell subset and absolute number of splenic Tfh cells. (C) Representative contour plots and histograms from flow cytometry analysis of IL-17-, IFN-γ-, and IL-4-expressing CD4⁺ T cells in B6 and c1(70-100) mice. Splenocytes were stimulated with PMA and ionomycin in the presence of GolgiStop for 4 h, and then fixed, stained with anti-CD3 and -CD4, permeabilized, and stained with anti-cytokine Ab. Thick lines outline the regions used to gate CD4⁺CD3⁺ T cells. For histograms, the percentage of cells staining positively for each cytokine is indicated. (D) Scatterplots showing the percentages of cytokine-producing cells as a proportion of the CD4⁺ T cell population. Horizontal lines indicate the mean of each group examined. Significance levels were determined by one-way ANOVA with Dunns’ post-test. The p values for significant differences between B6 and congenic mouse strains are shown with **p<0.01, ***p<0.001. Bars with p values above denote significant differences between congenic strains.
Figure 2.4. c1 congenic mice exhibit increased production of cytokines secreted by Tfh, Th1 and Th17 populations. Splenic CD4+ T cells were purified from 4-mo-old B6, c1(96-100), c1(88-100), and c1(70-100) mice using negative selection and were cultured with plate-bound anti-CD3 antibody in the presence of anti-CD28 for 48 h. Culture supernatants were assayed for cytokine production in triplicate with the levels of IL-2, IL-4, IL-17, and IFN-γ being determined using a cytokine bead array, and for IL-21 by ELISA. Each point represents the determination from an individual mouse. Horizontal lines indicate the mean for each population examined. Significance levels were determined by one-way ANOVA with Dunns’ post-test. The p values for significant differences between B6 and congenic mouse strains are shown with *p<0.05, **p<0.01, ***p<0.001. Bars with p values above denote significant differences between congenic strains.
Figure 2.5. Identification of cytokine-producing T cell subsets in c1 congenic mice. Freshly isolated splenocytes from 4-mo-old mice were stained with anti-CD3, -CD4, -CXCR5, and -PD1, permeabilized and then stained for intracellular IL-17 and IFN-γ production (as
described in Figure 2.3) with the addition of IL-21R/Fc chimera to detect IL-21 production. (A) Representative contour plots gated on CD3+CD4+ T cells from B6 and c1(70-100) mice are shown on the left for each strain. The regions used to define the Tfh and conventional (non-Tfh) cells are shown. Numbers indicate the proportion of each cell subset in the gated population. To the right are contour plots showing representative results for cytokine staining. The quadrants used to identify positively staining cells are shown. (B) Scatterplots showing the absolute number of Tfh, and non-Tfh cells producing IL-21 (top), IL-17 (middle), and IFN-γ (bottom). Each point represents the determination from an individual mouse. Horizontal lines indicate the mean for each population examined. (C) Splenic sections from 4-mo-old B6, c1(96-100), c1(88-00), and c1(70-100) mice were stained with FITC anti-IgM (Green), biotinylated-PNA followed by 7-amino-4-methylcoumarin-3-acetic acid-conjugated streptavidin (Blue), PE anti-IL-17 (Yellow) and allophycocyanin anti-CD4 (Purple). Arrows indicate the location of IL-17 producing CD4+ T cells within T cell areas for each mouse strain. Note that the increased numbers of IL-17-producing CD4+ T cells (white dots) in c1(70-100) mice are located predominantly in the T cell zone and not the GC. Magnification = ×10. The scale bar indicates 100 µm. (D) Scatter plot showing the number of IL-17-producing CD4+ T cells within the T cell zone. Each point represents the average number of IL-17-producing cells per T cell zone for an individual mouse, with 5-7 T cell zones being counted per mouse. Significance levels were determined by one-way ANOVA with Dunns’ post-test. The p values for significant differences between B6 and congenic mouse strains are shown with *p<0.05, **p<0.01, ***p<0.001. Bars with p values above denote significant differences between congenic strains.
2.4.2 *Intrinsic skewing of the immune system towards increased generation of Tfh, Th17 and Th1 cell subsets in c1 congenic mice*

To determine whether the increased production of IL-21, IL-17, and IFN-γ in c1 congenic mice was a consequence of the breakdown in tolerance to nuclear antigens, or resulted from intrinsically altered immune function leading to skewed Tfh, Th17 and Th1 development, we investigated the immune response to OVA as a representative exogenous antigen. Young pre-autoimmune 8-wk-old B6 and c1 congenic mice were immunized i.p. with OVA emulsified in CFA, using PBS emulsified in CFA as a control. The mice were sacrificed 14 days later and the proportions of various T cell subsets and GC B cells were examined. Consistent with our previous results (Figures 2.1 & 2.3), there was a progressive increase in the proportion of Tfh and GC B cells corresponding to increasing size of the NZB c1 interval, following OVA-CFA immunization (Figure 2.6 A). No significant differences were observed with PBS-CFA immunization. To assess the cytokine profile of the OVA-specific T cells, splenocytes isolated from OVA-primed mice were re-stimulated *in-vitro* with OVA for 72 h. Cytokine levels were measured in tissue culture supernatants and the amount of OVA-specific cytokine production was determined by subtracting cytokine production in the absence of OVA. As seen in 4-mo-old unimmunized mice, there were progressive increases in IFN-γ, IL-17, and IL-21 production with increasing length of the NZB c1 interval (Figure 2.6 B). Thus, the immune system in c1 congenic mice appears to be intrinsically skewed toward increased production of Th1, Th17, and Tfh cytokines, regardless of the specificity of the antigen.
Figure 2.6. Enhanced differentiation of pro-inflammatory T cell subsets in c1 congenic mice following OVA immunization. 8-wk-old mice were injected i.p. with OVA or PBS in CFA. The proportions of splenic Tfh cells (CD4⁺CD44hiCD62LloCXCR5hiPD1hi) and splenic GC B cells (B220⁺Fas⁺PNAhi) were determined by flow cytometry 2 wks later. (A) Scatterplots showing the proportion of Tfh and GC B cells as a proportion of the CD4⁺ T cell and B220⁺ B cell populations, respectively. (B) Scatterplots showing the amount of cytokine produced by OVA-primed splenocytes re-stimulated in-vitro with OVA for 72h. Assays were performed in triplicate and the levels of secreted cytokines measured by ELISA or cytokine bead array (see Methods). Each data point represents the mean of the triplicate with background cytokine production in the absence of antigen subtracted. Horizontal lines indicate the mean of each group examined. Significance levels were determined by one-way ANOVA with Dunns’ post-test. The p values for significant differences between B6 and congenic mouse strains are shown with *p<0.05, **p<0.01, ***p<0.001.
2.4.3 *Altered T cell differentiation in c1 congenic mice results from defects affecting T and non-T cell function*

To determine the immune defects that lead to the increased differentiation of CD4+ T cells into Th1, Th17 and Tfh cells in c1 congenic mice, several approaches were used. In the first approach, naïve T cells from the spleens of 8-wk-old pre-autoimmune mice were isolated and induced to differentiate into various T cell subsets using cocktails of cytokines and mAbs (see Materials and Methods). Under Th0 conditions there was minimal differentiation of either B6 or c1 congenic T cells into IL-21- (<0.21%), IL-17- (<0.12%), IFN-γ- (<0.82%), or IL-4- (<0.41%) secreting cells with similar levels seen for all mouse strains (Figure 2.7 A). In contrast, under Th1-inducing conditions, all c1 congenic mice demonstrated increased differentiation to IFN-γ-secreting cells compared to B6 mice (Figure 2.7 B), suggesting that a genetic locus in the NZB c1 96-100 cM interval promotes differentiation of this cell subset. Using Th17-inducing conditions, both c1(88-100) and c1(70-100) naïve T cells demonstrated equivalently increased differentiation to IL-17-producing cells compared to B6 or c1(96-100) T cells. Thus, a genetic locus located within the NZB c1 88-96 interval alters T cell function to promote IL-17 secretion. In contrast, similar proportions of Th2 and IL-21-producing cells were seen for all mouse strains tested under their respective cytokine inducing conditions, suggesting that the increased proportions of IL-21 producing cells seen *in-vivo* in c1 congenic mice do not arise from a T cell functional defect.
Figure 2.7. Increased differentiation of naïve CD4+ T cells from c1 congenic mice to Th17 and Th1 cells *in-vitro*. Naïve T cells from 8-wk-old mice were stimulated under Th0, Th1, Th2, Th17, and IL-21-producing polarizing conditions and cytokine production quantified 5 days later by flow cytometry (see Methods). (A) Representative contour plots gated on CD3+CD4+ T cells from B6 and c1(70-100) mice. For each polarizing condition, plots for relevant cytokine production under Th0 conditions (-) and polarizing conditions (+) are shown. The quadrants used to define positively and negatively staining cells are indicated. (B) Scatterplots showing the percentage of T cells that are IL-21-producing (Tfh), Th17, Th1 and Th2 cells, under relevant polarizing conditions. Horizontal lines indicate the mean for each population examined. Significance levels were determined by one-way ANOVA with Dunns’ post-test. The *p* values for significant differences between B6 and congenic mouse strains are shown with *p*<0.05, **p*<0.01, ***p*<0.001. Bars with *p* values above denote significant differences between congenic strains.
The second approach used to examine the altered T cell differentiation in c1 congenic mice was adoptive transfer of B6 or congenic T cells into B6 or congenic recipients in a reciprocal fashion. To facilitate these investigations, an OT-II TCR transgene (Tg) with specificity for OVA/A\textsuperscript{b}, was crossed onto the various mouse backgrounds. Naïve CD4\textsuperscript{+} T cells were then purified from the spleens of young 8-wk-old OT-II TCR Tg B6 and c1 congenic mice, and injected into the tail vein of 8-wk-old B6.Thy1.1 or c1(70-100).Thy1.1 mice. Mice were then immunized with OVA and the differentiation of naïve OT-II T cells into various Th cell subsets determined by flow cytometry, gating on the transferred Thy1.2\textsuperscript{+} population (Figure 2.8 A). These results confirmed the in-vitro Th differentiation results, showing that the enhanced IFN-\gamma- and IL-17-, but not IL-21-, secreting cell differentiation arises in part from intrinsic T cell defects localizing to the NZB c1 96-100 and 88-96 intervals, respectively (Figures 2.8 B&C). However, there was also an important role for the environment in the increased differentiation that was observed, because OT-II T cells from all of the mouse strains demonstrated enhanced differentiation to Tfh, Th1, Th17, and IL-21-secreting populations when transferred into c1(70-100).Thy1.1 mice. Indeed, only minimal non-significant increases in the proportion of IFN-\gamma- and IL-17-secreting cells for the relevant c1 congenic T cells were seen upon adoptive transfer into B6 mice. This finding suggests that the increased differentiation of these T cell subsets in c1 congenic mice is critically dependent upon cellular and/or cytokine cues that are not provided by the B6 environment.
Figure 2.8. *Intrinsic T cell functional defects together with altered environmental cues promote the enhanced differentiation of OVA-specific T cell subsets in congenic mice.* Naïve T cells from OT-II TCR Tg mice were transferred into pre-autoimmune B6.Thy1.1 or c1(70-100).Thy1.1 mice, that were subsequently immunized with OVA in CFA. Mice were sacrificed 2 wks later and the proportion of transferred T cells differentiating to various T cell subsets was examined by flow cytometry. (A) Representative contour plots following transfer of B6 or c1(70-100) OT-II cells into c1(70-100).Thy1.1 mice. Transferred cells were identified by staining the splenocytes from recipient mice with anti-Thy1.2 mAb. Tfh cells were identified by gating on the CD4+CD44^hi^PD1^hi^CXCR5^hi^ cells (indicated by boxed regions) within this subset. Cytokine-producing cells were identified as outlined in Figures 1 and 2, and the Methods. Scatter plots of the proportion of (B) Tfh and (C) cytokine-producing cells within the transferred T cell population. The open and closed symbols represent cells transferred into B6 or c1(70-100) recipient mice. Horizontal lines indicate the mean of each group examined. Significance levels were determined by one-way ANOVA with Dunns’ post-test. The p values for significant differences between B6 and congenic mouse strains are shown with *p<0.05.
2.4.4 DC from c1(88-100) and c1(70-100) mice demonstrate altered function that promotes differentiation of pro-inflammatory T cell subsets

Cues from DC play an important role in directing the differentiation of T cells following Ag challenge. We therefore contrasted the ability of DC from the various strains of mice to direct the differentiation of OT-II T cells when cultured with low concentrations of OVA in-vitro. To this end, bone marrow was isolated from 8-12-wk-old B6 and c1 congenic mice and cultured with FLT3L for 7 days to expand DC. This yielded bone marrow DC (BMDC) that were ~25% plasmacytoid DC (pDC) and ~30% myeloid DC (mDC) with the remaining cells having an indeterminate phenotype. Similar proportions and numbers of DC were seen for all strains. The BMDC were then co-cultured with OVA 323-339 peptide and OT-II T cells from B6 or c1 congenic mice for 4 days in the presence of GM-CSF. As shown in Figure 2.10A, BMDC from c1(70-100) mice demonstrated a significantly enhanced ability to induce differentiation of Th1 cells compared to those from B6 and c1(96-100) mice, and similar non-significant trends were seen for c1(88-100) mice and for Th17 and Tfh cell differentiation. For Th1 cells this increased induction was only seen for OT-II cells from the congenic mouse strains, indicating that T cell and DC defects must interact with each other to induce this phenotype. Similar findings were observed for Th17 cells, where differences between induction of Th17 differentiation by B6 and c1(88-100) or c1(70-100) DC were most pronounced for c1(88-100) and c1(70-100) T cells. Thus, BMDC from congenic mice appear to be able to direct differentiation of T cells in a way that is compatible with the altered differentiation that is observed in-vivo. Experiments using BMDC expanded with GM-CSF or whole splenocytes (Figure 2.9 A) as antigen-presenting cells yielded very similar results for comparison of B6 and c1(70-100) cells.
Figure 2.9. Splenic mDC from c1(70-100) congenic showed increased production of IL-6 and IL-12, and induce enhanced T cell differentiation *in vitro*. Freshly isolated splenocytes from 5-6-wk old B6.Thy1.1 or c1(70-100).Thy1.1 were co-cultured with OVA peptide and purified naïve CD4+ T cells from OT-II TCR Tg B6 and c1(70-100) mice. On day 3, the cells were re-stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop or GolgiPlug, and analyzed by flow cytometry for cell surface DC (CD11c, CD11b, B220), B cell (CD19, B220) or T cell (CD3, CD4) markers and intracellular cytokine levels. (A) Scatterplots showing the percentage of IL-21-, IL-17- and IFN-γ-producing T cells. Results are clustered in groups based on the strain of the T cells (top of the figure) with the strain of origin of the splenocytes shown at the bottom of the figure. (B) Scatterplots showing the percentage of B cells and mDC producing IL-12 and IL-6. (C) Scatterplot showing the proportion of B cells and mDC within the splenic population. Horizontal lines indicate the mean.
To further explore the mechanisms by which BMDC from c1(88-100) and c1(70-100) mice promote differentiation of pro-inflammatory T cell subsets, mDC activation and cytokine production was examined in the co-culture system. Consistent with their ability to enhance differentiation of Th1 and to a lesser extent Th17 and Tfh cells, BMDC from c1(88-100) and c1(70-100) mice secreted elevated levels of IL-12 and IL-6 which achieved statistical significance for c1(70-100) mice (Figure 2.10 B). Similar findings were seen for c1(70-100) splenic mDC when whole splenocytes were used as antigen-presenting cells (Figure 2.9 B). A trend to elevated levels of MHC class II and B7.2 were also seen on c1(88-100) mDC, which were further increased on c1(70-100) mDC (Figure 2.10 B). Notably, these changes were independent of the strain of T cells with which the DC were co-cultured (data not shown). In contrast to the data observed for mDC, no differences in cytokine secretion or activation were seen between strains for pDC in the culture (Figure 2.10 C).

In lupus, the immune response is focused on nuclear antigens contained in apoptotic debris. We have previously shown that in NZB c1 congenic mice there is a breach of tolerance to these antigens, resulting in spontaneous priming of histone-reactive T cells (93). This observation suggests that the DC in these mice may have processed and presented nuclear antigens. Since these nuclear antigens can activate TLRs, enhancing DC activation and presentation, we investigated whether the BMDC abnormality in c1 congenic mice leads to altered TLR responses. Consistent with the results of our co-culture experiments, mDC from c1(88-100) and c1(70-100) mice demonstrated significantly increased intracellular levels of IL-12 and a trend to increased intracellular levels of IL-6 in response to CpG stimulation (Figure 2.11 A&B). Increased intracellular levels of IL-6 were also observed for c1(70-100) derived mDC following stimulation with Poly(I:C). No differences were seen for the secretion of IFN-
α, IL-23 or TNF-α (Figure 2.11 C&D), nor were differences seen for MHC-II or B7.2 expression following TLR stimulation (data not shown). These findings indicate that the altered DC function in c1(88-100) and c1(70-100) mice also affects the response to certain TLR signals.
Figure 2.10. Myeloid DC from c1(88-100) and c1(70-100) mice demonstrate altered function and an enhanced ability to induce differentiation of Th1 cells. BMDC from 8–12 wk-old mice were expanded with FLT3L for 7 days and then co-cultured with OVA peptide and purified naïve CD4+ T cells from OT-II TCR Tg mice. On day 4, the cells were re-stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop or GolgiPlug, and analyzed by flow cytometry for cell surface DC (CD11c, CD11b, B220, MHC-II, B7.2) or T cell (CD3, CD4) markers and intracellular cytokine levels. (A) Scatterplots showing the percentage of IL-21-, IL-17- and IFN-γ-producing T cells. Results are clustered in groups based on the strain of T cells (top of the figure) with the DC strain shown at the bottom of the figure. Scatterplots showing the percentage of CD11c+CD11b+B220− mDC (B) and CD11c+CD11b−B220+ pDC (C) expressing elevated levels of MHCII and B7.2, or IL-6 and IL-12. Results with the different strains of T cells have been pooled as no differences were noted between strains. Horizontal lines indicate the mean. Significance levels were determined by one-way ANOVA with Dunns’ post-test. The p values for significant differences between B6 and congenic mouse strains are shown with *p<0.05, **p<0.01, ***p<0.001. Bars with p values above denote significant differences between congenic strains.
A

Gated on CD11c+ cells

B6

cf(70-100).

Gated on mDC cells

B

B

B

Media
Imiquimod
Poly(I:C)
CpG
LPS

MFI of IL-12 (mDC)

MFI of IL-6 (mDC)

P<0.05

P<0.01

P<0.05
Figure 2.11. Altered production of IL-6 and/or IL-12 by myeloid DC from c1(88-100) and c1(70-100) mice following stimulation with TLR ligands. BMDC from 8-12 week-old mice were expanded with FLT3L and then cultured in the presence or absence of Imiquimod R837, Poly(I:C), CpG 2216, or LPS for 18h with GolgiStop (for IL-12) or GolgiPlug (for IL-6) being added for the last 6 h. The cells were then stained as outlined in Figure 5 and the
Methods. (A) Left panel shows representative dot plots indicating the regions used to gate B220⁺CD11b⁻ pDC (top left box) and B220⁺CD11b⁺ mDC (bottom right box) within the CD11c⁺ population. Shown to the right are representative histogram plots of IL-6 and IL-12 for B6 (solid grey) and c1(70-100) mice (black line) in unstimulated (Media) and stimulated (Poly (I:C) for IL-6 or CpG 2216 for IL-12) conditions. (B) Scatterplots showing the MFI for IL-6 and IL-12 expression on mDC. (C) IFN-α and IL-23 levels in the culture supernatants of BMDC as measured by ELISA. (D) MFI for TNF-α expression in mDC. Horizontal lines indicate the mean. Significance levels were determined by one-way ANOVA with Dunns’ post-test. The \( p \) values for significant differences between B6 and congenic mouse strains are shown with *\( p < 0.05 \), **\( p < 0.01 \). Bars with \( p \) values above denote significant differences between congenic strains.
2.5 Discussion

In this paper, we show that differences in the severity of renal disease that we have previously published for a series of NZB c1 congenic mouse strains correlate with the expansion of pro-inflammatory T cell subsets including, Th1, Th17, and Tfh cells. These findings are compatible with previous work suggesting that these cells populations drive pathogenic autoantibody production and/or inflammatory changes in the kidney (207, 217, 232, 237).

Mice with the shortest NZB c1 interval, c1(96-100), showed no evidence of Tfh cell expansion. Consistent with the lack of Tfh cell expansion, the major increase in cytokine production in these mice appeared to arise from the conventional T cell subset, where slight increases in the number of IFN-γ, IL-17, and IL-21 secreting cells were seen. In contrast, c1(88-100) mice demonstrated significant increases in Tfh and IFN-γ-, IL-17-, and IL-21-producing T cells. While our experiments do not allow us to definitively conclude which expanded cell populations are driving the increased disease severity in these mice, we have previously shown that IgG2a Ab and complement are deposited in their kidneys (94), implicating IFN-γ-producing T cells in this process. However, it is likely that Tfh also play a role, since we have shown that CD40L is necessary for production of GC and nephritis in NZB and c1 mice ((241) and unpublished observations). Notably, the Tfh cells in c1(88-100) mice do not produce significant amounts of IL-17. This finding contrasts with those observed in BXD2 lupus-prone mice, where substantial numbers of IL-17-producing Tfh cells were seen and introduction of an IL-17R knockout attenuated disease (232).
Although c1(70-100) mice showed trends to increased numbers and/or proportions of IFN-\(\gamma\)-, IL-17-, and IL-21-secreting cells compared to c1(88-100) mice, the most marked differences were for IL-21-secreting cells, particularly those that also secreted IFN-\(\gamma\) (data not shown). Since the severity of renal disease in our mice is closely associated with IgG deposition in the kidney (94), it is likely that these changes augment kidney disease through enhanced selection of pathogenic IgG in the GC. Nevertheless, we cannot exclude a possible role for the IFN-\(\gamma\) or IL-17-producing cells in either providing extra-follicular T cell help or directly impacting inflammation in the kidney in these mice. In keeping with the latter possibility, both IFN-\(\gamma\)- and IL-17-secreting cells have been found infiltrating the kidneys of c1(70-100) mice (unpublished observations).

Experiments in young pre-autoimmune mice immunized with a representative foreign antigen recapitulated the same types of pro-inflammatory cell expansions as seen in older mice. Using various approaches it was demonstrated that alterations in both T cell and DC function contribute to the changes observed. Enhanced differentiation of naïve T cells to IFN-\(\gamma\)-producing cells was localized to the NZB c1 96-100 interval. Although the genetic polymorphism leading to this altered differentiation has not been definitively identified, it is likely that it arises from polymorphisms in the Slam family. The NZB c1 96-100 interval overlaps with the region containing the Sle1b lupus susceptibility allele identified in NZM2410 mice. NZB, NZM2410, and a variety of other autoimmune mouse strains share the same Slam allele which differs from that of B6 mice at genetic loci for multiple Slam family members. The top candidate gene in this interval is Ly108, which encodes a self-ligating membrane glycoprotein that has at least three alternatively spliced isoforms differing in their cytoplasmic domains (84, 114, 242). In autoimmune mouse strains, expression of Ly108-1 is increased,
whereas Ly108-2 expression is decreased and Ly108-H1 is absent (84, 86). Since stimulation with anti-Ly108 antibody induces T cell IFN-γ secretion, it is possible that increased expression of the Ly108-1 variant in c1(96-100) T cells leads to enhanced differentiation to IFN-γ-producing cells (243). Alternatively, the absence of Ly108-H1 expression could produce this phenotype, since introduction of a BAC Ly108-H1 transgene onto mice lacking this isoform was associated with reduced numbers of IFN-γ-producing CD4+ T cells in-vivo (86). Recently, B6.Sle1b mice were found to have an expansion of Tfh cells that was first detectable at 6-8 months of age (a time point 2 months later than the mice examined in our study) and that could be corrected with the Ly108-H1 transgene (244). Since c1(96-100) also lack the Ly108-H1 isoform (our own unpublished observations), the findings reported herein suggest that this expansion does not arise from an intrinsic T cell functional defect.

A second T cell functional change, leading to increased generation of IL-17-producing T cells, appeared to require the NZB c1 88-96 interval. It is currently unknown whether this functional change arises solely from genetic polymorphisms located within the c1 88-96 interval or results from interaction between polymorphisms in the c1 96-100 and 88-96 intervals. Candidate genes within the 88-96 interval include: the retinoid X receptor gamma (Rxrg), a member of the RXR family of nuclear receptors which have been shown to modify the balance between Th1 and Th2 cells (245-247); and Pre-B cell leukemia homeobox 1 (Pbx1) that has also been shown to influence T cell differentiation. Recently, increased T cell expression of a novel splice isoform of this gene, Pbx1-d, was found in B6 congenic mice with the Sle1a lupus susceptibility locus (83).

Despite the presence of intrinsic T cell functional abnormalities in c1 congenic mice, this does not appear to be sufficient to induce altered spontaneous T cell differentiation in vivo,
pointing to a critical role of environmental cues in the induction of the abnormal differentiation of T cells in c1(88-100) and c1(70-100) mice. Nevertheless, T cell abnormalities also appear to be essential, as B6 T cells did not differentiate efficiently to IFN-γ- or IL-17-producing cells following adoptive transfer into c1(70-100) mice *in-vivo*. Our experiments suggest that altered DC function provides one of the environmental cues that enhances pro-inflammatory T cell differentiation in c1(88-100) and c1(70-100) mice. DC from c1(70-100), and to a lesser extent c1(88-100), mice demonstrated enhanced production of IL-12 which has been shown to promote differentiation of naïve T cells to Th1 and Tfh phenotypes (248-250) and increased levels of IL-6 which has been shown to promote differentiation of naïve T cells to Th17 and Tfh phenotypes (211, 225, 251, 252). Following these initial interactions, IL-21 production by activated Th17 and Tfh cells could act in an autocrine manner to further direct DC-primed CD4+ T cells to become Th17 and Tfh cells (226, 228, 253), resulting in a positive feedback loop. It is likely that the enhanced ability of mDC from c1(70-100) and, to a lesser extent, c1(88-100) mice to upregulate MHC class II and B7.2 in response to T-DC interaction further augments the differentiation of pro-inflammatory T cell subsets in these mice.

In summary, we demonstrate that T cell and DC defects, derived from several genetic loci, synergize to convert preclinical disease to fatal GN by leading to expansion of pro-inflammatory T cells. This data joins an increasing body of data from the study of congenic mouse strains demonstrating that impact of individual genetic loci on immune function and autoimmunity is highly dependent upon their genetic/immunologic context (182, 254-256). These studies have important implications for the study of human autoimmune disease, in that they provide an explanation for how the presence of a susceptibility locus in the family members of a patient with autoimmune disease can be compatible with relatively normal
immune function, whereas the same locus in the patient leads to profoundly altered immune function. Thus, the identification of individuals with an increased likelihood of developing autoimmune disease must necessarily involve characterization of multiple interacting genetic loci.
Chapter 3

Identification of the SLAM Adapter Molecule EAT-2 as a Lupus Susceptibility Gene that Acts through Impaired Negative Regulation of Dendritic Cell Signaling

Nafiseh Talaei*,†, Tao Yu‡, Kieran Manion*,†, Rod Bremner‡,§, and Joan E. Wither*†¶

*Arthritis Centre of Excellence, Toronto Western Research Institute, University Health Network, Toronto, Ontario; †Lunenfeld Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Ontario; and Departments of ‡Immunology, §Laboratory Medicine and Pathology, and ¶Medicine, University of Toronto, Toronto, Ontario

All experiments were performed by N. Talaei. T. Yu assisted in generation of results for Figure 3.1 E, K. Manion provided help with mouse breeding and the bone marrow isolations.

3.1 Abstract

We have previously shown that C57BL/6 congenic mice with an introgressed homozygous 70-100 cM chromosome 1 interval (c1(70-100)) from the lupus-prone New Zealand Black (NZB) mouse, develop high titres of antinuclear antibodies and severe glomerulonephritis. Using sub-congenic mice, we found that a genetic locus in the 88-96 cM region was associated with altered dendritic cell (DC) function and synergized with T cell functional defects to promote expansion of pathogenic pro-inflammatory T cell subsets. Here we show that the promoter region of the NZB gene, encoding the SLAM signaling pathway adapter molecule EAT-2, is polymorphic and that this results in a ~70% reduction in EAT-2 in DCs. Silencing of the EAT-2 gene in DCs that lacked this polymorphism led to increased production of IL-12 and enhanced differentiation of T cells to a Th1 phenotype in T cell - DC co-cultures, reproducing the phenotype observed for c1(70-100) DC. SLAM signaling has been previously shown to inhibit production of IL-12 by CD40L-activated DCs. Consistent with a role for EAT-2 in this inhibition, knock-down of EAT-2 resulted in increased production of IL-12 by CD40-stimulated DCs. Assessment of downstream signaling following CD40 crosslinking in the presence or absence of SLAM crosslinking revealed that SLAM co-engagement blocked activation of p38 MAP Kinase and JNK signaling pathways in DCs, which was reversed in DCs with the NZB EAT-2 allele. We conclude that EAT-2 negatively regulates cytokine production in DCs downstream of SLAM engagement and that a genetic polymorphism that disturbs this process promotes the development of lupus.
3.2 Introduction

SLE is a complex genetic disease in which multiple susceptibility loci interact with each other to produce the autoimmune phenotype (257). Given the outbred nature of the human population, investigation of mouse strains that spontaneously develop a lupus-like illness, where genetic regions containing susceptibility loci can be isolated for study by crossing them onto a non-autoimmune background, have played an important role in the identification of genetic polymorphisms and immune abnormalities that lead to lupus, together with how they interact to promote disease development (6, 67).

Studies in our laboratory have focused on the New Zealand Black (NZB) mouse that develops a lupus-like disease characterized by production of anti-ssDNA and –RBC antibodies (Abs), leading to hemolytic anemia and mild glomerulonephritis (GN) (8, 74). To identify the immune mechanisms leading to disease in these mice, we produced and characterized a series of C57BL/6 (B6) congenic mouse strains with homozygous intervals containing susceptibility loci derived from this mouse strain. In previous work, we showed that mice with an NZB interval extending from 70 cM (125.6 Mb) to 100 cM (179.8 Mb) on chromosome 1 (c1(70-100)) developed a severe lupus phenotype, with fatal GN developing in 40% of animals by 8 months of age (94). By examining sub-congenic mice with shorter overlapping intervals, we found that at least 3 genetic loci were required to produce this phenotype (94). Congenic mice with the shortest NZB interval, extending from 96 to 100 cM (170.8-179.8 Mb; c1(96-100)) and containing the polymorphic SLAM locus, had mild subclinical autoimmunity characterized by production of anti-ssDNA Abs in the absence of anti-dsDNA Abs or kidney disease. In contrast, mice with longer NZB intervals demonstrated a progressive increase in the levels of anti-dsDNA Abs and the severity of renal disease that paralleled the size of the NZB interval.
Further investigation of the immune mechanisms accompanying this increase in disease severity revealed the presence of intrinsic T cell and dendritic cell (DC) functional abnormalities that led to enhanced expansion of pro-inflammatory T cell subsets, including Th1, Th17 and T follicular helper (TFH) cells (258).

In this study, we sought to identify the genetic polymorphism that leads to altered DC function in NZB c1 congenic mice. We have previously shown that the gene leading to this altered function is localized to the 88-96 cM interval and that it leads to increased production of IL-12 and IL-6 by bone marrow-derived dendritic cells (BMDC) in T cell – DC co-culture experiments in vitro (258). Here we show that there is a promoter polymorphism in NZB mice that leads to reduced levels of the SLAM adapter molecule EWS-activated transcript 2 (EAT-2, also called sh2d1b1) within the 88-96 interval, and that knockdown of EAT-2 in BMDC of mice that lack this polymorphism reproduces the altered DC phenotype. We further demonstrate that BMDC from mice with the NZB polymorphism or that have EAT-2 knocked down produce increased amounts of cytokines in response to CD40 crosslinking and that EAT-2 plays an important role in the negative regulation of CD40 signaling following SLAM engagement.
3.3 Materials and Methods

3.3.1 Ethics statement

Mice were housed in a Canadian Council on Animal Care approved facility at the Toronto Western Research Institute, part of the University Health Network. All mice used and experiments performed in this study were approved by the Animal Care Committee of the University Health Network (Animal Use Protocol #123).

3.3.2 Mice

B6 mice were purchased from Taconic (Germantown, NY). B6.OT-II TCR transgenic (Tg) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and then bred in our facility. Congenic mouse strains with homozygous NZB chromosome 1 intervals of varying length were generated as previously described (94). OT-II TCR Tg congenic mice were produced by polymorphic marker assisted backcrossing. Only female mice were examined and all mice were specific-pathogen free.

3.3.3 Flow cytometry

RBC-depleted splenocytes (0.5 x 10⁶) were incubated with mouse IgG (Sigma-Aldrich, St. Louis, MO) for 15 min and then stained with various combinations of directly-conjugated mAbs followed by allophycocyanin- or PerCP-Cy5.5-conjugated streptavidin (BD Biosciences (San Diego, CA) and BioLegend (San Diego, CA), respectively). Dead cells were excluded by staining with 0.6 μg/ml propidium iodide (PI, Sigma-Aldrich). Events were acquired using a LSR II instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR). The following directly conjugated mAbs were purchased from BD Biosciences.
or BioLegend: Biotin-conjugated anti-CD4 (RM4-5), -B220(RA3-6B2), and -CD86 (B7.2; GL1); PE-conjugated anti-CD44 (IM7), B7.2 (GL1), CD40(3/23), CD84(mCD84.7) and -IA/IE (M5/114.15.2); PE-Cy7-conjugated anti-CD44 (IM7) and -CD11c (N418); Pacific Blue-conjugated anti-B220 (RA3-6B2) and -CD4 (RM4-5); PerCP-Cy5.5-conjugated anti-B220 (RA3-6B2); Allophycocyanin-conjugated anti-CD150 (TC15-12F12.2); and FITC-conjugated anti-CD11b (M1/70), -CD62L (MEL-14). All isotype controls were obtained from BD Biosciences or BioLegend.

3.3.4 BMDC isolation and expansion

Bone marrow cells were isolated by flushing the femurs of 6–10-wk-old congenic or B6 mice. RBC were lysed and then the cells were re-suspended at 10⁶ cells/mL in complete RPMI medium (10% FCS + additives) and cultured for 8 days with 40 ng/ml recombinant mouse GM-CSF (R&D Systems, Minneapolis, MN) to expand BMDC. Non-adherent cells, harvested at day 8, were used for all experiments.

3.3.5 Measurement of EAT-2 mRNA expression in BMDC

RNA was purified from BMDC using a Total RNA Mini Kit (FroggaBio, Toronto, ON, Canada), and converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Forster City, CA). Quantitative real-time PCR (qRT-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 as follows: β-actin (TTGCTGACAGGATGCAGAAG, and GTACTTGCGCTCAGGAGGAG); EAT-2 (ATGCTCATACTCCAAGAACGA, and TCAGCTCTAACTCCATTCTTCT). Gene
expression was analyzed using the relative standard curve method and was normalized to $\beta$-
actin expression.

3.3.6 Promoter sequencing and Luciferase Assay

Genomic DNA was extracted from the kidneys of B6 and NZB mice. Briefly, the tissue was digested with lysis buffer (100 mM NaCl, 10mM Tris-Hcl, 25 mM EDTA pH=8, 0.5% Sodium Dodecyl Sulfate, 0.1 mg/ml Proteinase K), extracted with phenol:chloroform:isomyl alcohol (25:24:1), and the DNA precipitated from the aqueous layer by addition of ammonium acetate and ethanol. The EAT-2 promoter region extending from -300 base pair (bp) to +159 bp from the initiation codon was amplified using forward TTAGTGCTGCTGCTCCAGCC and reverse CCTGCATGAAGATGAGGATAC primers, cloned into a TOPO 2.1 vector (Invitrogen), and sequenced (ACGT Corp., Toronto, Canada). Reporter constructs were generated by cloning the EAT-2 promoter region into a firefly luciferase reporter plasmid, pREP4–Luc (259). 300 bp fragments containing the EAT-2 promoter region were prepared using forward, AGATCTGATTTTAGTGTGCTGCTCCA (BglII site underlined) and reverse GCGGCCGCAGGATTGTTATGTTTAAAACCG (NotI site underlined) primers and then directionally cloned into the BglII/NotI sites of pREP4–Luc to generate EAT-2 reporter plasmids (pREP4–B6-EAT2–Luc and pREP4–NZB-EAT2–Luc). Correct integration of the fragments was confirmed by sequencing. BMDC were prepared from 6-8-wk-old B6 mice, as described above, and $5 \times 10^6$ cells were transfected with 5 $\mu$g of EAT-2 reporter plasmid or pREP4–Luc empty vector (plus 50 ng of a Renilla luciferase plasmid (Promega, USA) as a control for transfection efficiency) by electroporation using 400 V, 200 $\mu$F and 1000 ohms (Bio-Rad, Hercules, CA). After 24 or 48 h of incubation with GM-CSF (40 ng/ml), the cells were
harvested and washed, and extracts were prepared using a Dual Luciferase Assay Kit (Promega). Luciferase activity was measured by luminometer (PerkinElmer Victor3) and the results expressed as Firefly luciferase activity normalized to Renilla luciferase activity.

3.3.7 Transfection of BMDC with EAT-2 siRNAs

A total of $5 \times 10^6$ BMDC were electroporated, as above, with siGENOME Non-Targeting Control siRNAs (scrambled control) or siGENOME SMARTpool Sh2d1b1(EAT-2) siRNAs (GE Healthcare Dharmacon, Lafayette, CO) at a final concentration of 4 µM. siGLO Green transfection indicator (100 nM, GE Healthcare Dharmacon) was used to measure the transfection efficiency. After transfection, cells were incubated in RPMI 1640 containing 20% FCS plus additives for 24 h. The BMDC were washed before use in subsequent experiments.

3.3.8 In vitro culture of transfected BMDC and OVA-specific T cells

Naïve CD4+ T cells (CD4+CD62Lhi) were isolated from the spleens of 6-12-wk-old B6 or congenic OT-II Tg mice using a mouse CD4+CD62L+ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). 2 x 10^5 OT.II naïve CD4+ T cells were then co-cultured with 2 x 10^4 transfected BMDC and OVA 323-339 peptide (GenScript, Piscataway, NJ) in the presence of 5 ng/ml recombinant mouse GM-CSF (R&D Systems) for 2 days. Prior to measurement of intracellular cytokines, PMA (50 ng/ml) and ionomycin (1 µg/ml) together with GolgiPlug or GolgiStop (BD Biosciences) were added for the last 4 h before harvesting. The cells were then stained for cell surface DC (CD11c, CD11b) or T cell (CD4) markers, fixed, permeabilized, and stained for detection of intracellular cytokines, as previously described (258). For T cells, intracellular cytokines were detected using allophycocyanin-anti–IFN-γ (XMG1.2), Alexa
Fluor® 488-anti-IL-17A (TC11-18H10), and PE-anti-IL-21 (mhalx21), all from e-Bioscience. For DC, intracellular cytokines were detected using allophycocyanin-conjugated anti-IL-12 (C15.6) or Alexa Fluor® 488-conjugated anti-IL-6 (MP5-20F3), both from BD Biosciences. BD Horizon fixable viability stain 450 (FVS450) was used to exclude dead cells.

3.3.9 Western Blots

BMDC were lysed in RIPA buffer (150mM NaCl, 50mM Tris, 1mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS), with phenylmethylsulfonyl fluoride and Protease Inhibitor Cocktail (Santa Cruz Biotechnology Inc, Dallas, TX) added immediately prior to use. Lysates were incubated on ice for 5 min, centrifuged for 10 min, and then the supernatant harvested. Protein concentration was determined using a BSA Protein Assay Kit (Thermo Scientific, Rockford, IL). Samples were then separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk and probed with anti-mouse-EAT-2 (Santa Cruz Biotechnology) or anti-mouse-β-actin (Cell Signaling Technology) for 2 h at room temperature (RT) or overnight at 4°C. Immunoreactive bands were detected using horseradish peroxidase–conjugated secondary antibody and visualized using an enhanced chemiluminescent system (Thermo Fisher scientific, Waltham, MA).

3.3.10 BMDC CD40 stimulation

BMDC (1 × 10^6) were stimulated with 10 μg/ml anti-mouse CD40 (3/23, BD Biosciences) for 24 h, with GolgiPlug or GolgiStop (BD Biosciences) added for the last 4h before harvesting. The cells were then stained for cell surface DC markers (CD11c, CD11b),
fixed, permeabilized, and stained for detection of intracellular cytokines as described previously.

3.3.11 Phos-flow

BM-derived DCs (BMDC) ($1 \times 10^6$) were resuspended in 200 µl RPMI (1% FCS plus additives) and incubated for 30 min at 4°C in the presence of 5 µg/ml anti-CD150 (TC15-12F12.2, BioLegend) or anti-CD84 (mCD84.7, BioLegend). After washing to remove unbound antibody, the cells were warmed to 37°C for 10 min, and incubated for 2 min in the presence or absence of 10 µg/ml anti-CD40, and 5 µg/ml goat anti-rat IgG (BioLegend) or goat anti-Armenian Hamster IgG (Jackson Immunoresearch Labs, West Grove, PA) to crosslink CD150 and CD84, respectively. The cells were then fixed with 2% paraformaldehyde and frozen at -80°C. Following thawing, the cells were stained to detect cell surface molecules, permeabilized in 70% methanol, re-suspended in Perm/Wash™ buffer (BD Biosciences), and stained with antibodies directed against intracellular signaling molecules as follows: PE-anti-phospho (p)-JNK (pT183/pY185; N9-66) and -p-P38 MAPK (pT180/pY182; 36/p38), all from BD Biosciences.

3.3.12 Immunoprecipitation

BMDC ($1 \times 10^7$) were resuspended in 200 µl RPMI (1% FCS plus additives) and incubated for 30 min at 4°C in the presence of 5 µg/ml anti-CD150 or anti-CD84. After washing, the cells were warmed to 37°C for 10 min, and incubated for 2 min with 5 µg/ml goat anti-rat IgG or goat anti-Armenian Hamster IgG to crosslink CD150 and CD84, respectively. Lysis buffer was then added to each sample and the lysate pre-cleared by incubation with
Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) for 1 h at 4°C. After centrifugation, the solution was incubated with 5 µg/ml anti-CD150 (BioLegend) or anti-CD84 (BioLegend) for 4 h or overnight at 4°C, followed by incubation with 20 µL of Protein A/G PLUS-Agarose for 2 h at 4°C. Immunoprecipitated proteins were then collected by boiling in electrophoresis sample buffer (Santa Cruz Biotechnology), separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk or 5% BSA and probed with anti-phosphotyrosine antibody (clone 4G10, EMD Millipore, MA, USA), anti-CD150, or anti-CD84 antibodies for 2 hours at room temperature or overnight at 4°C. Immunoreactive bands were visualized as described above.

3.3.13 Statistical analysis

Comparisons of differences between groups of mice for continuous data were performed using a one-way ANOVA followed by Dunns’ post-test for multiple comparisons or a Mann-Whitney nonparametric test when two groups were compared. Differences with p values of < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad software (La Jolla, CA, USA).
3.4 Results

3.4.1 A genetic polymorphism in the promoter region of EAT-2 in NZB c1 congenic mice results in altered expression of EAT-2

Our previous work suggested that there was a lupus susceptibility gene in the 88-96 cM interval of NZB c1 that acts by altering DC function. Of the 80 genes within this interval, there are 18 protein coding genes, with EAT-2 being the only gene reported to be expressed in DCs. As the coding regions of B6 and NZB EAT-2 alleles had been sequenced and did not differ (260), we investigated whether there were altered levels of expression of EAT-2 in the DCs of mice with the NZB c1 88-96 cM interval. RNA was isolated from BMDCs following culture in media containing recombinant GM-CSF for 8 days, and the expression of EAT-2 mRNA was evaluated using qRT-PCR. As shown in Figure 3.1A, the levels of EAT-2 were reduced by ~70% in BMDC from c1(70-100) and c1(88-100) mice, both of which have NZB c1 intervals containing the NZB EAT-2 variant, compared to c1(96-100) and B6 mice, which do not. Western blots confirmed that the protein levels of EAT-2 were similarly reduced in c1(88-100) and c1(70-100) BMDC (Figure 3.1 B&C).

To determine whether the altered expression of EAT-2 in these mice resulted from a promoter polymorphism, DNA from B6 and NZB mice was sequenced. This revealed two mutations in the EAT-2 promoter region of mice with the NZB variant that were just upstream of the initiation codon. Computational analysis suggested that the mutated regions were potential binding sites for the transcription factors CREB1 and Klf4, and that the mutations in NZB mice resulted in disruption of these binding sites (Figure 3.1D).

To confirm that the promoter polymorphism in the NZB EAT-2 gene leads to reduced gene expression, the B6 and NZB EAT-2 promoter regions were cloned into a luciferase reporter
construct (pREP4-Luc) (259). The constructs were then transfected into B6 BMDCs and luciferase activity was measured. There was significantly reduced luciferase activity with the construct containing the NZB promoter region compared to the B6-derived EAT-2 promoter (Figure 3.1 E). Thus, the reduced levels of EAT-2 in c1(88-100) and c1(70-100) mice appear to arise from a promoter polymorphism that leads to reduced RNA expression in DCs.

3.4.2 Knockdown of EAT-2 in BMDCs from B6 and c1(96-100) mice recapitulates the c1(70-100) phenotype.

We have previously shown that BMDCs from congenic mice with a NZB 88-96 cM region demonstrate enhanced secretion of IL-12 and IL-6 when co-cultured with OVA 323-339 peptide and naïve TCR transgenic OT-II OVA-specific T cells, which was associated with significantly enhanced differentiation of the OT-II cells to Th1 and a trend to increased generation of Th17 and Tfh cells (258). As shown in Figure 3.2 A, c1(70-100) BMDCs produced increased amounts of IL-12 in co-culture with T cells from all strains tested, whereas increased amounts of IL-6 were only produced in co-cultures with c1(96-100) or c1(70-100) T cells, suggesting that genetic interactions between the NZB loci in the 88-96 and 96-100 regions augment IL-6 production.

To determine whether knockdown of EAT-2 alters DC function in a way compatible with these functional changes, siRNAs targeting this gene were introduced into B6 and c1(96-100) BMDCs, with scrambled non-targeting siRNAs acting as a control. Transfection efficiency, as determined using the siGLO Green transfection indicator, was 85-90% (Figure 3.3).
Introduction of targeting siRNAs into BMDCs resulted in a ~85% reduction in EAT-2 RNA expression (Figure 3.2B), leading to a ~80% reduction in EAT-2 protein levels (Figure 3.2 C). 24 h after transfection, BMDCs were co-cultured with naïve OT-II T cells from B6 or c1(96-100) mice, together with OVA peptide for 2 days. Representative results are shown in Figure 3.2 D&E, with pooled results from several experiments shown in Figure 3.2 F&G. As seen for c1(70-100) BMDCs, knockdown of the EAT-2 gene in BMDCs from both B6 and c1(96-100) strains resulted in increased production of IL-12 compared to scrambled control, and this was associated with increased differentiation of OVA-specific T cells from both B6 and c1(96-100) mice to a Th1 phenotype (Figure 3.2 F). Findings similar to those observed for c1(70-100) BMDCs were also seen for IL-6, where knockdown of EAT-2 in BMDCs led to increased IL-6 production only in co-cultures with c1(96-100) OT-II T cells (Figure 3.2 G). However, this was only observed when EAT-2 was reduced in c1(96-100) BMDCs, indicating that a NZB 96-100 genetic polymorphism is also required in BMDCs for increased generation of IL-6. This was associated with enhanced production of IL-21 (Figure 3.2 G) but not IL-17 in co-cultured c1(96-100) OT-II T cells. Knockdown of EAT-2 did not affect production of IL-12 and IL-6 by DCs in response to TLR ligands (data not shown). In summary, EAT-2 appears to be negatively regulating cytokine production in DCs and the low levels of EAT-2 in c1(70-100) and c1(88-100) mice may contribute to the increased production of IL-12 and IL-6 that we have previously observed for their DCs.
Figure 3.1 A NZB EAT-2 polymorphism leads to decreased expression of EAT-2 in BMDCs. (A) Scatterplot showing EAT-2 mRNA expression in BMDCs from various mouse strains. BMDCs from 6–10-wk-old B6 and c1 congenic mice were expanded with GM-CSF for 8 days before harvesting. EAT-2 mRNA expression levels were measured using qRT-PCR and normalized to β-actin mRNA expression. Each point represents the determination from an individual mouse. Horizontal lines represent the mean for each group. (B) Western blot analysis of lysates prepared from BMDCs of B6 and c1 congenic mice. The top panel shows representative blots for EAT-2 (20 kDa) and the bottom panel for β-actin. All samples were run on the same gel, with white lines indicating where the lanes were joined to produce the final image. Numbers below represent levels, as quantified using a densitometer. (C) Scatterplot showing the relative densities of EAT-2 protein bands normalized to β-actin. The $p$ values for
significant differences between B6 controls and various congenic mice are shown, *p<0.05, **p<0.01. (D) Sequencing results from NZB and B6 genomic DNA. Highlighted sequences indicate differences in the *EAT*-2 promoter region in the NZB as compared to the B6 mouse strain. (E) B6 BMDCs were transfected with *EAT*-2 reporter plasmids (pREP4–B6-EAT2–Luc and pREP4–NZB-EAT2–Luc) or pREP4–Luc empty vectors. Shown is the activity of the indicated firefly luciferase reporter in transfected BMDCs, at 0, 24, or 48 h, following incubation with GM-CSF. The firefly luciferase activity has been normalized to that of Renilla luciferase. Data represent the means ± standard deviation of triplicate samples from three different experiments. The p value for significant differences between different luciferase vectors is shown, **p<0.01.
Figure 3.2 Knockdown of EAT-2 leads to increased production of IL-12 by DCs and increased differentiation of OT-II T cells to Th1 cells in vitro. (A) BMDCs from 6–10-wk-old B6 and c1 congenic mice were expanded with GM-CSF for 8 days, and then were co-cultured with OVA peptide and purified naïve CD4+ T cells from OT-II TCR Tg mice for 48 h. The cells were then re-stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop or GolgiPlug, and analyzed by flow cytometry following staining for DC cell surface markers (CD11c, CD11b) and intracellular cytokine levels. Results are clustered in groups based on the strain of T cell (top of panel) with the DC strains of origin shown at the bottom of each plot. Scatterplots show the MFI for expression of IL-12 (top) or IL-6 (bottom) in DCs (gated as CD11b+CD11c+). (B) The levels of EAT-2 mRNA expression in BMDCs 48 hr after electroporation with non-targeting control (scrambled) or EAT-2-specific (siRNA) siRNAs. EAT-2 mRNA expression levels were measured using qRT-PCR and normalized to β-actin mRNA expression. (C) Western blots showing EAT-2 protein and β-actin levels before and after transfection with EAT-2 specific siRNAs. (D-G) BMDCs were electroporated, as outlined above, and 24 h later co-cultured with OVA peptide and purified naïve CD4+ T cells from OT-II TCR Tg mice for 48 h. The cells were then re-stimulated as described above, and analyzed by flow cytometry for cell surface DC (CD11c, CD11b) or T cell (CD4) markers and intracellular cytokine levels. (D & E) Representative histograms derived from c1(96-100) (D) DCs or (E) T
cells showing intracellular (D) IL-12 and IL-6 or (E) IFN-γ and IL-21 staining levels. Grey shaded histograms represent cells from co-cultures with BMDC transfected with scrambled control; thick line histograms represent cells from co-cultures with BMDCs transfected with EAT-2-specific siRNAs. The thin line represents the unstained control. (F) Scatterplots showing the MFI for DC IL-12 expression (top) and the percentage of IFN-γ-producing T cells (bottom). (G) Scatterplots showing the MFI for DC IL-6 expression (top) and the percentage of IL-21-producing T cells (bottom). Each point represents the determination from an individual mouse. Horizontal lines represent the mean for each group. Asterisks indicate the significance level for comparisons between different mouse strains (* p<0.05, ** p<0.01).
Figure 3.3 BMDC transfection efficiency as determined by siGLO green indicator. BMDCs from 6-10-wk old B6 and c1 congenic mice were expanded with GM-CSF for 8 days, then were electroporated with or without (control) 100 nM siGLO green, and analyzed by flow cytometry 24h after transfection. Representative dot plots showing transfection efficiency, with transfected cells gated in boxes and the percentage of cells transfected shown at the top right of the plot.
3.4.3 Reduced levels of EAT-2 in c1 congenic DC result in enhanced IL-12 production in response to CD40 signaling

Activation of DCs by CD40 engagement with CD40 ligand expressed on activated T cells is one of the major pathways leading to DC maturation and cytokine production. Previous work has shown that SLAM/SLAM interactions inhibit production of IL-12 and IL-6 by CD40 ligand activated DCs (261). EAT-2 interacts with phosphorylated SLAM family receptors, such as CD84, CD150, LY9 and CD244 in immune cells, and in NK cells has been shown to be a negative regulator of cellular activation downstream of the SLAM receptor 2B4 (127, 262, 263). We therefore questioned whether one of the mechanisms by which reduced EAT-2 leads to enhanced cytokine production was through impaired negative regulation of CD40-stimulation in DCs. Consistent with this concept, CD40 crosslinking resulted in significantly enhanced production of IL-12 for c1(88-100) and c1(70-100) BMDC compared to B6 and c1(96-100) BMDCs (Figure 3.4 A). No differences were seen in IL-6 production between the strains. To confirm that it was the reduced levels of EAT-2 in c1(88-100) and c1(70-100) BMDC that were leading to enhanced production of IL-12 in these cells, EAT-2 was knocked down in B6 and c1(96-100) BMDCs. As shown in Figure 3.4 B, introduction of siRNAs for EAT-2 resulted in significant augmentation of IL-12, but not IL-6 production, following CD40 stimulation, with comparable levels observed for B6 and c1(96-100) BMDC. Consistent with an important role for CD40 signaling in the enhanced production of IL-12 and IFN-γ that was seen in OT-II T cell co-cultures with DC, treatment with anti-CD40L led to a marked reduction in the levels of IL-12- and IFN-γ-producing cells and the differences between EAT-2 sufficient and deficient DC were lost (Figure 3.4 C). Taken together, these findings suggest that the enhanced production of
IL-12 in c1 congenic mice with the NZB EAT-2 polymorphism results from impaired SLAM-mediated negative regulation of CD40 signaling.
Figure 3.4 Increased production of IL-12 by anti-CD40-stimulated BMDC from c1(88-100) and c1(70-100) mice is recapitulated by EAT-2 knockdown in control cells. BMDCs were stimulated with 10μg/ml anti-CD40 mAb for 24h and the levels of IL-12 or IL-6 production examined by flow cytometry as described in the Materials and Methods section. (A) Scatterplots showing the MFI for IL-12 (top) and IL-6 (bottom) expression after CD40 stimulation. (B) Scatter plots showing MFI for IL-12 and IL-6 expression in transfected stimulated DCs. BMDCs from B6 or c1(96-100) mice were transfected with scrambled control or EAT-2 specific siRNAs (as described in Figure 3.2) and stimulated with anti-CD40, as described above. (C) Scatterplots showing the MFI for DC IL-12 expression (top) and the percentage of IFN-γ-producing T cells (bottom). BMDC from B6 or c1(96-100) mice were transfected with scrambled control or EAT-2 specific siRNAs (as described in Figure 2) and 24 h later co-cultured with OVA peptide and purified naïve CD4+ T cells from OT-II TCR Tg mice ±10μg/ml anti-CD40L mAb for 48 h. The cells were then re-stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop or GolgiPlug, and then analyzed by flow cytometry for cell surface DC (CD11c, CD11b) or T cell (CD4) markers and intracellular cytokine levels. Each point represents the determination from an individual mouse. Horizontal lines represent the mean for each group. Asterisks indicate the significance level for comparisons between different mouse strains (* p<0.05).
CD40-mediated signal transduction induces activation of several well-characterized signal transduction pathways including p38 Mitogen-activated protein kinase (MAPK) and Jun amino-terminal kinase (JNK), which have been shown to promote IL-12 production downstream of CD40 in DCs (264). To determine whether EAT-2 negatively regulates this pathway, DCs from all mouse strains were stimulated with anti-CD40 alone or in tandem with activating anti-SLAM antibodies. There were no differences between the mouse strains in the levels of CD40 expression (Figure 3.5). As CD150 engagement has been previously shown to inhibit CD40-mediated activation of DCs, anti-CD150 antibodies were used to activate the SLAM pathway (261). However, examination of CD150 expression on GM-CSF-expanded BMDCs revealed significantly reduced levels of CD150 on c1 congenic BMDCs compared to B6 BMDCs (Figure 3.6 A&B). Therefore, as an additional control for potential expression level related effects, we also examined cytokine secretion following anti-CD84 crosslinking. CD84 was chosen because it is expressed at high levels on DCs and previous reports indicated CD84 negatively regulates the cytokine secretion induced by FceRI receptor stimulation in mast cells (261, 265). In contrast to CD150 expression, CD84 expression was elevated on c1 congenic BMDCs relative to B6 BMDCs (Figure 3.6 A&B).

As shown in Figure 3.6 C, crosslinking of CD84 or CD150 led to similar levels of phosphorylation on a per molecule basis for all mouse strains, confirming that the antibodies are activating and that there are no differences between strains in the ability of the antibodies to induce phosphorylation of these molecules. Similarly, stimulation with anti-CD40 alone led to equivalently enhanced phosphorylation of MAPK-P38 and JNK in all mouse strains (Figure 3.5 D). In contrast, CD40 stimulation in tandem with anti-CD84 or -CD150 crosslinking led to significant inhibition of JNK and p38 MAPK phosphorylation in B6 and c1(96-100) BMDCs, as
compared to CD40 stimulation alone, whereas phosphorylation of these signaling molecules was unchanged from CD40 stimulation alone in c1(70-100) BMDCs. These findings suggest that EAT-2 acts upstream of the MAPK pathway to inhibit production of IL-12, and that the reduced levels of EAT-2 in c1(88-100) and c1(70-100) BMDCs lead to impaired inhibition of this pathway.

Previous studies suggest that activation of the PI 3-kinase (PI3K) pathway blocks IL-12 production following CD40 engagement (266). Since EAT-2 has been shown to activate PI3K in NK cells (267), we questioned whether this was also the case for DC. To investigate this possibility we assessed phosphorylation of AKT, which is commonly used as an indicator of activation of this pathway (268). As shown in Figure 3.4D, p-AKT was increased when CD40 was crosslinked in tandem with SLAM in BMDC with the B6 EAT-2 allele, and this was absent in c1(70-100) BMDC. These findings are consistent with the possibility that EAT-2 is acting to inhibit IL-12 production by activating the PI3K pathway.
Figure 3.5 BMDCs express the same level of CD40 in all mouse strains. BMDCs from 6–10-wk old B6 and c1 congenic mice were expanded with GM-CSF for 8 days, and then analyzed by flow cytometry for expression of CD40 on DCs (gated as CD11b+CD11c+). Scatterplots showing the percentage of CD40 expression on BMDCs for each mouse strain.
Figure 3.6. SLAM-mediated inhibition of signaling downstream of CD40 is deficient in BMDC from c1(70-100) mice. BMDC from 6–10-wk-old B6 and c1 congenic mice were expanded with GM-CSF for 8 days, and then analyzed by flow cytometry for expression of CD150 or CD84 expression on DC (gated as CD11b<sup>+</sup>CD11c<sup>+</sup>). (A) Representative histograms show BMDC derived from B6 (thick lines) and c1(96-100) mice (thin lines). Gray shaded histograms represent background of unstained cells. (B) Scatterplots showing the MFI for CD150 or CD84 expression on BMDC for each mouse strain. (C) BMDC from B6, c1(96-100) and c1(70-100) were stimulated with anti-CD150 (Top) or anti-CD84 (Bottom) for 2 min. The cells were lysed, immunoprecipitated with anti-CD150 and CD84, and then immunoblotted with the indicated antibodies. For each IP, all samples were run on the same gel, with white lines indicating where the lanes were rearranged and joined to produce the final image. (D) Scatterplots showing the MFI levels for p-MAPK (p38), p-JNK, and p-AKT in BMDC following CD40 stimulation in the presence or absence of CD84 or CD150 crosslinking. B6 or c1 congenic BMDC were stimulated with anti-CD40 alone or in tandem with anti-CD84 or -CD150. The cells were then fixed, stained, and gated as outlined in the Materials and Methods section. The p values for significant differences between B6 and congenic mouse strains are shown (*p<0.05, **p<0.01) and were determined by one way ANOVA test followed by Dunns' post-test.
3.5 Discussion

We previously showed that full expression of the autoimmune phenotype in NZB c1 congenic mice results from the interaction between at least 3 genetic loci (94). While a breach of tolerance to nuclear antigens was seen in mice with the NZB c1 96-100 cM interval, pathogenic autoimmunity required the presence of additional defects localized to the NZB 70-96 cM interval that were associated with marked expansion of pro-inflammatory T cell subsets including Th1, Th17, and Tfh cells in vivo. As the same abnormal expansion could be observed for foreign antigen-specific responses, we were able to dissect the functional abnormalities leading to the altered T cell differentiation in NZB c1 congenic mice, showing that T cell and DC functional alterations interacted to produce this phenotype. Despite the presence of intrinsic T cell functional abnormalities in c1 congenic mice leading to enhanced differentiation of their naïve T cells in response to Th1 and Th17 polarizing stimuli, maximal expansion of pro-inflammatory T cell subsets in an antigenic response required interaction of these T cells with DCs from mice with a NZB c1 interval containing the 88-100 cM region (258). Mice with this interval were shown to have functionally altered DCs that produced increased amounts of IL-12 and IL-6 in co-culture experiments with T cells. Here we provide several lines of evidence indicating that this altered function results from a promoter polymorphism in the NZB EAT-2 gene that leads to reduced levels of EAT-2 protein expression.

Although the SLAM family of receptors modulates immune responses through several adapters including SAP, EAT-2 and ERT, EAT-2 is the only known SLAM-associated adapter protein expressed in DCs (263). EAT-2 is also found in NK cells (263, 269), where it has been shown to play an important role in the regulation of NK cell function mediated by SLAM family members (269). Although some controversy exists surrounding whether EAT-2 can
promote or inhibit NK cell function depending on the upstream SLAM molecule that is engaged (130, 270, 271). EAT-2 has been shown to be required for the inhibition of NK cell function that is mediated by 2B4 (127). Based on this observation and previous findings indicating that engagement of CD150 leads to impaired production of cytokines by DCs in response to CD40 engagement (261), we hypothesized that the reduced levels of Eat-2 in c1 congenic BMDC lead to impaired negative regulation of this signaling pathway, resulting in augmented production of cytokines. Consistent with this hypothesis, we show that knocking down the levels of EAT-2 in DCs leads to augmented cytokine production similar to that observed for the NZB EAT-2 polymorphism in T-DC co-culture experiments and also leads to significantly increased production of IL-12 in response to CD40 engagement.

In contrast to the results for IL-12, knockdown of EAT-2 did not lead to enhanced production of IL-6 with CD40 stimulation alone. This finding, together with the observation that enhanced IL-6 production was only seen for c1(96-100) BMDCs with EAT-2 knocked down that had been co-cultured with c1 congenic T cells, suggests that there are additional signals derived from NZB polymorphisms in the 96-100 interval that are required to promote IL-6 production. Although several polymorphic genes have been described in this interval, the most likely candidates to produce these differences are members of the SLAM family itself. NZB mice are reported to have a similar SLAM allele to NZM mice, although minor sequence variations were noted (85). The presence of this SLAM allele was found to correlate with susceptibility to SLE and was associated with altered expression of several SLAM family members (85). Subsequent experiments have largely focused upon the role of Ly108 in this phenotype, where differences in the expression of several splice variants of this gene have been noted. In autoimmune mouse strains, expression of Ly108-1 is increased, whereas Ly108-2
expression is decreased and Ly108-H1 is absent (84, 86). Unpublished experiments from our laboratory have confirmed similarly altered expression of these splice variants in c1 congenic T cells. Notably, absence of a Ly108-H1 splice variant has been shown to promote differentiation of IFN-γ-producing T cells (86). Therefore, it is likely that this contributes to the enhanced production of IFN-γ by c1 congenic T cells in T cell - DC co-cultures (see Figure 2F). Although IFN-γ induces IL-6 production in monocytes and other cell types (272, 273), which could contribute to enhanced production of IL-6 in BMDCs, this is insufficient to explain the increased IL-6 production in the T-DC co-cultures, because this is not observed for B6 BMDCs. Thus, additional NZB-derived functional differences in BMDCs must contribute to this phenotype. In this connection, it is interesting to note that there are several SLAM family members that are differentially expressed in c1 congenic and B6 BMDCs and T cells. As shown in this paper, GM-CSF-expanded c1 congenic BMDCs have decreased levels of CD150 and increased levels of CD84, and similar changes are seen on CD4+ T cells. Previous experiments indicate that in the absence of CD150, IFN-γ production by T cells is increased (274), raising the possibility that the low levels of CD150 could contribute to the enhanced IFN-γ production observed for c1 congenic T cells. As the majority of SLAM-molecules, including CD150 and CD84, are homophilic receptors, low levels of CD150 on BMDCs could further enhance IFN-γ production by c1 congenic CD4+ T cells, leading to further increases in IL-6. This finding is consistent with the observation that knocking down EAT-2 in c1(96-100) BMDCs leads to a greater enhancement in IFN-γ production than knockdown of EAT-2 in B6 BMDCs (see Figure 2).

It has been reported that CD40-mediated activation of the p38 MAP Kinase and JNK signaling pathways promotes IL-12 production by DCs (275-278) and that PI 3-kinase (PI3K)
has an inhibitory effect on this production by blocking these pathways through an unknown mechanism (266). Since engagement of CD150 or CS1 has been shown to trigger PI3K activation (267, 279-281) and this activation is mediated by EAT-2 in NK cells (267), we hypothesized that EAT-2 acts downstream of SLAM engagement to decrease IL-12 production in BMDC by blocking activation of the p38 MAPK and JNK pathways. Consistent with this possibility, engagement of CD150 or CD84 led to significantly reduced levels of p38 MAPK and JNK phosphorylation following CD40 stimulation of BMDCs, which was not seen for c1 congenic mouse strains with the NZB EAT-2 polymorphism. Currently, the precise molecules that are recruited through EAT-2 activation downstream of the SLAM receptors in DCs and mechanisms by which they inhibit p38 MAPK and JNK activation downstream of the CD40 receptor are unknown.

Our findings further highlight the importance of the SLAM signaling pathway in the regulation of autoimmunity. SLAM signaling plays an important role in the fine-tuning of a diverse array of immunologic functions including: 1) cytokine production by T cells, macrophages, neutrophils and DCs (263, 274, 282); 2) B-T cell adhesion (283); 3) B cell signaling and tolerance (84, 284, 285); 4) NK T cell development (118); 5) NK function (118); 6) T cell differentiation, in particular to Tfh cells (274, 286); and 7) platelet aggregation (118). Mice bearing the autoimmune-associated SLAM allele have been shown to have disturbances in several of these processes, such as enhanced differentiation of their T cells to Th1 and Tfh cells, decreased immature B cell receptor editing and apoptosis, impaired GC B cell tolerance, and altered pDC function with increased production of IL-10 and IFN-α (84, 91, 274, 285, 286). The majority of these differences have been ascribed to the altered splicing of Ly108 observed in these mice, although a role for altered expression of other polymorphic members of the
SLAM family cannot be excluded. In this report, we provide evidence for a second murine lupus-susceptibility gene that affects the SLAM signaling pathway. Although our report focuses upon the role of this polymorphism in DCs, it is also possible that this polymorphism leads to altered function other cell populations, such as B cells and NK cells that are known to express EAT-2 (263).

It is likely that disturbed SLAM signaling also plays a role in the immunologic derangement associated with human lupus. Altered expression of several SLAM family members has been noted on the T cells, NK cells, and pDCs of SLE patients (287-290). In T cells, this altered expression is associated with an enhanced ability of the SLE T cells to differentiate to IL-17-producing cells with Th17 polarizing stimuli (288). While there is some data suggesting that there are lupus genetic risk variants in the SLAM signaling pathway (113, 291, 292), it is currently unclear whether this altered expression arises from these variants or from the pro-inflammatory milieu associated with the disease.

In summary, our findings indicate that the SLAM pathway, and in particular EAT-2, appears to play a crucial role in limiting cytokine secretion in myeloid DCs. In this context, it is notable that expression of all SLAM molecules examined increased following CD40 stimulation, suggesting the presence of a negative feedback loop in which up-regulation of SLAM molecules following DC activation blocks cytokine secretion, limiting the expansion of pro-inflammatory T cell subsets. As demonstrated in this and our previous paper (258), the consequence of this impaired negative regulation is increased provision of help for pathogenic auto-antibody production, promoting the conversion of sub-clinical autoimmunity to fatal lupus nephritis.
Chapter 4

General Discussion and Future Directions

The main focus of this thesis was to identify the lupus susceptibility genes located within the 70 and 100 cM of NZB chromosome and to define the immune mechanisms by which they act to promote disease. In work done prior to my experiments, Dr. Wither’s laboratory had shown that mice with a NZB c1 interval extending from 70-100 cM (c1(70-100)) develop a severe lupus phenotype, with high titers of anti-dsDNA Abs and GN, leading to the death of ~40% of the mice by 8 months of age. This phenotype appeared to result from at least 3 genetic loci, as indicated by progressively attenuated disease in mice with NZB c1 intervals extending from 88- or 96-100 cM (94).

In Chapter 2, I characterized the immune changes in a series of sub-congenic mice with varying lengths of intervals derived from the NZB 70-100 cM region that differed in the severity of disease. I showed that the disease severity in these mice paralleled the expansion of pro-inflammatory T cell subsets, specifically Th1, Th17, and Tfh cells. I further demonstrated that this expansion could be recapitulated following immunization of pre-autoimmune mice with an exogenous antigen. This T cell skewing resulted from a combination of immune cell functional abnormalities in congenic mice that localized to different regions within the c1(70-100) interval. Naïve T cell functional abnormalities that promote differentiation/expansion of IFN-γ- and IL-17- producing cells localized to the 96-100 and 88-96 intervals, respectively, whereas DC functional abnormalities that promote expansion of all the pro-inflammatory T cell subsets localized to the 88-96 and 70-88 intervals. Notably, altered DC function appeared to
play a critical role in this expansion because in the absence of DC abnormalities, minimal expansion of pro-inflammatory T cell subsets was seen.

In addition to understanding the mechanism of disease in c1 congenic mice, it is important to identify the genes that mediate these functional abnormalities. While the T cell functional changes leading to expansion of IFN-γ-producing cells appear to map to the 96-100 region, it is currently unknown whether T cell functional changes leading to expansion of IL-17-producing cells in c1 congenic mice arise solely from genetic polymorphisms located within the c1 88-96 interval, or result from interactions between polymorphisms in the c1 96-100 and 88-96 intervals.

Within the 88-100 interval, Ly108, Pbx1 and Rxr-γ are three attractive candidate genes that theoretically may promote altered T cell function in c1 congenic mice. To determine whether these candidate genes are associated with altered generation of Th1 and Th17 cells, I have examined the expression level of Ly108, Pbx1 and Rxr-γ in naïve CD4+ splenic T cells and in unstimulated or anti-CD3/CD28 stimulated cells using qRT-PCR.

Ly108 is within the 96-100 interval and is a member of the SLAM/CD2 gene family that has at least three alternatively spliced isoforms (85). In autoimmune mouse strains, expression of Ly108.1 is increased, whereas Ly108.2 expression is decreased and Ly108-H1 is absent. Looking at the RNA expression levels of different Ly108 isoforms showed that consistent with previous findings for the NZM2410 mouse strain (which has the NZW SLAM allele), stimulated T cells from c1 congenic mice had increased expression of the Ly108.1 isoform and lacked Ly108-H1 (Figure 4.1). However in contrast to previous reports for NZM2410 mice, differences in expression of the Ly108.1 or Ly108.2 isoforms were only noted following activation of the cells (Figure 4.1).
Figure 4.1 Expression levels of the different *Ly108* isoforms in c1 congenic mice. Naive CD4$^+$ splenic T cells were isolated from 8wk old B6 and c1 congenic mice. RNA was purified from unstimulated or anti-CD3/CD28 stimulated cells and gene expression was contrasted between strains using qRT-PCR. Scatterplot showing the relative expression of *Ly108* normalized to β-actin expression. The *p* values for significant differences between B6 controls and various congenic mice are shown, *p<0.05, **p<0.01.
In subsequent experiments to delineate the role of Ly108 in Th1 and Th17 differentiation, we had knocked down the Ly108 gene using siRNA in naïve T cells from c1 congenic and B6 mice and then cultured the cells under Th1 and Th17 polarizing conditions. Knock down of Ly108 reduced Th1 and Th17 differentiation in both c1 congenic and B6 mouse T cells. These findings are compatible with a role for Ly108 in augmenting IFN-γ production (Figure 4.2).

In summary, these preliminary results suggest that Ly108 isoforms are differentially expressed in our sub-congenic mice, however to further investigate the role of these differences in promoting the functional changes in c1 congenic T cells it will be necessary to perform further experiments. Experiments such as introducing a retroviral or lentiviral vector mediating overexpression of different splice variants, in particular the Ly108-H1 isoform, would help to more precisely evaluate the role of each splice variant in abnormal Th1 and Th17 differentiation in c1 congenic mice. However, we cannot rule out the possibility that polymorphisms in other SLAM molecules contribute to the differences seen in Th1 and Th17 differentiation. Knock down of Ly108 in c1(96-100) and c1(70-100) naïve T cells did not reduce production of IFN-γ to B6 levels (Figure 4.2), suggesting that another polymorphism in the 96-100 interval may contribute to the increased IFN-γ seen in congenic mouse T cells. Polymorphisms in other SLAM molecules such as CD84, CD150, CD244 and CD48 are the most likely candidates for this difference.
Figure 4.2 Knock down of Ly108 leads to reduced Th1 and Th17 differentiation in both c1 congenic and B6 mouse T cells. Naive CD4+ splenic T cells were isolated from 8wk old B6 and c1 congenic mice and transfected with Ly108 siRNA or scramble control. The cells were cultured under Th1 or Th17 polarizing conditions (according to figure 2.3). On day 3, the cells were re-stimulated with PMA and ionomycin for 4 h in the presence of GolgiPlug, and analyzed by flow cytometry for cell surface T cell (CD3, CD4) markers and intracellular cytokine levels. Scatterplots showing percentage of IFN-γ and IL-17 -producing T cells. Asterisks indicate the significance level for comparisons between different mouse strains (* p<0.05, ** p<0.01).
The second candidate gene, Pre-leukemia homeobox (Pbx) 1, is located within the 88-96 region and has three different isoforms: a, b and d. The Pbx1-d isoform is expressed at high levels in the NZM2410 lupus-prone mouse strain and has been shown to have a dominant negative effect on Pbx1 function, resulting in altered T cell activation and tolerance (83). Expression levels of Pbx1-a, b and d isoforms in naïve and activated T cells did not differ between c1 congenic and B6 mice (data not shown). However, these preliminary data cannot rule out possible expression differences for the Pbx1-d isoform in c1 congenic mice, as we only evaluated the expression levels in naïve T cells derived from 6-8 week old mice. Previous reports suggest that Pbx1 is upregulated in memory T cells and that the altered expression of Pbx1d is particularly apparent in the memory activated proportion of T cells from aged Sle1a mice. Consequently, evaluation of the expression levels of the Pbx1-d isoform in CD4+ T cells from aged c1 congenic mice, in particular the memory activated compartment, is necessary before this can be excluded as a candidate gene.

Pbx1 appears to play an important role in the control of T cell differentiation by the retinoic acid (RA) signaling pathway (83). RA induces differentiation of induced regulatory T cells (iTregs) and inhibits Th17 differentiation in the presence of TGFβ, by enhancing TGFβ-driven Smad3 signaling and inhibiting IL-6 and IL-23 expression, resulting in enhanced suppressive activity of murine (293-295) and human T cells (296, 297). Expression of the Pbx1-d isoform in Sle1a induces a defective CD4+ T cell response to RA and TGFβ under Th17-polarizing conditions, resulting in significantly reduced expansion of iTregs (298). Evaluation of the Sle1a transcriptional signature revealed defective Th17/Treg homeostasis in response to RA. Therefore, assessment of the CD4+ response to RA would help to identify whether this pathway is abnormal in c1(70-100) congenic mice. In particular, evaluation of T cell
differentiation in response to RA and TGFβ in Th17 polarization conditions would help to identify a potential role for *Pbx1d* in the increased differentiation of Th17 cells in c1 congenic mice.

The third candidate gene, which is located within the 88-96 interval, is Retinoid X receptor-γ (*Rxr-γ*). *Rxr-γ* belongs to the family of Retinoid X receptors (RXRs), which are members of the NR2B family of nuclear receptors and are common binding partners to many nuclear receptors, including PPARs (peroxisome proliferation/activation receptors). PPARs play essential roles in the regulation of T cell survival, activation and differentiation into the Th1, Th2, Th17, and Treg lineages (299). Recently, a member of the PPAR family, named PPAR gamma, has been identified as a Th17 differentiation regulator (300). Indeed, studies have shown that RXRs play a role in Th differentiation and T cell response modulation by modifying the balance of Th1 and Th2 cells (245, 246).

Although *Rxr-γ* appears to be an attractive candidate gene, expression levels of *Rxr-γ* in naïve T cells did not differ between B6 and c1 congenic mice at rest and only T cells from c1(70-100) mice showed lower expression after 24h stimulation with anti-CD3 and CD28 (Figure 4.3). Since *Rxr-γ* is polymorphic between the B6 and NZB mouse strains, and the recombination between B6 and NZB genetic material for c1(88-100) mice appears to be within this gene, further work will be required to clarify the role of this gene in T cell responses. This includes examination of the expression levels of different splice variants in naïve T cells from c1(88-100) and c1(70-100) mice and knock down of *RXR-γ* in T cells to determine its impact on T cell differentiation.
Figure 4.3 T cells from c1(70-100) mice show lower expression levels of Rxr-γ after 24h stimulation with anti-CD3 and -CD28. Naive CD4+ splenic T cells were isolated from 8wk old B6 and c1 congenic mice. RNA was purified from un-stimulated or anti-CD3/CD28-stimulated cells and gene expression was contrasted between strains using qRT-PCR. Bar graphs showing the relative expression of Rxr-γ normalized to β-actin expression. The p values for significant differences between B6 controls and various congenic mice are shown, *p<0.05.
Examining the potential link between the expansion of Th17 cells and altered expression levels of different \(Rxr-\gamma\) isoforms may reveal a potential mechanism for transcriptional regulation of Th17 differentiation by \(Rxr-\gamma\) through interaction with PPAR receptors. Understanding the mechanisms by which \(Rxr-\gamma\) controls Th17 differentiation will help to elucidate the function of this important receptor family during Th17 differentiation and may provide new targets for the treatment of Th17-dependent autoimmunity.

In Chapter 3, I identified the genetic polymorphism that leads to altered DC function in NZB chromosome 1 congenic mice. I showed that the promoter region of the NZB gene encoding the SLAM signaling pathway adapter molecule EAT-2 is polymorphic and that this results in a ~70% reduction in EAT-2 in DCs. Knockdown of \(EAT-2\) in BMDCs of mice that lacked this polymorphism reproduced the altered DC phenotype. I further demonstrated that BMDCs from mice with the NZB polymorphism, or that have \(EAT-2\) knocked down, produce increased amounts of cytokines in response to CD40 crosslinking and that \(EAT-2\) plays an important role in the negative regulation of CD40 signaling following SLAM engagement.

Our findings indicate that the SLAM pathway, and in particular EAT-2, appears to play a crucial role in limiting cytokine secretion in mDCs. In this context, it is notable that expression of all SLAM molecules examined increased following CD40 stimulation, suggesting the presence of a negative feedback loop in which up-regulation of SLAM molecules following DC activation blocks cytokine secretion, limiting the expansion of pro-inflammatory T cell subsets. The consequence of this impaired negative regulation is increased support for pathogenic auto-antibody production, converting the benign anti-nuclear antibody production seen in c1(96-100) mice to the severe life-threatening nephritis observed in c1(70-100) mice.
More work needs to be done to identify the other genetic polymorphisms within the 70-88 interval that augment the severity of disease in c1(70-100) mice. This interval contains over 200 genes, many of which have, or are predicted to have, immune functions. Of these, several have been shown to modify TLR responses in DCs, including: Map kinase activated protein kinase 2 (Mapkapk2), inhibitor of kappa B kinase epsilon (Ikbke), and DEAH box polypeptide nine (Dhx9). The gene encoding Ox40L (Tnfsf4) is also localized within this interval. This suggests a putative role for receptor/ligand interaction (OX40/OX40L) in enhancing the division and survival of T cells in c1(70-100) mice (301). OX40-OX40L interactions are implicated in the pathogenesis of human lupus as revealed by recent reports demonstrating a direct association between the severity of lupus nephritis and increased expression of OX40 on CD4+ T cells and enhanced serum levels of OX40L (302).

The findings outlined in this thesis provide important insights into how individual susceptibility loci, which alone produce modest changes in immune function, interact synergistically to profoundly alter immune functions, leading to progression from preclinical to pathogenic autoimmunity. By themselves, neither the T cell nor the DC functional alterations were sufficient to induce enhanced differentiation of pro-inflammatory T cell subsets. Thus, no single cell population and no single genetic locus in isolation is sufficient to produce clinical autoimmune disease in this model. Indeed, our studies of murine lupus outlined in this thesis have important implications for the study of human autoimmune disease, in that they provide an explanation for how genetic loci that are present in the family members of patients with autoimmune disease can be compatible with relatively normal immune function, whereas in patients they lead to profoundly altered immune function. The results also suggest that the impact of individual genetic loci on immune function is highly dependent upon their
genetic/immunologic context. Thus, the identification of individuals with an increased likelihood of developing autoimmune disease must necessarily involve characterization of multiple genetic elements acting in concert. Identification of susceptible genes may provide insights into the pathogenesis behind SLE and may lead to targeted therapies of lupus nephritis.


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and CD84-dependent NK cell functions in the C57BL/6 mouse. *J Immunol* 185:5683-5687.


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