Dissecting the Genetics and Function of CD5$^+$ B and invariant NKT cells using Congenic Lupus-Prone Mouse Strains

By

Yuriy Baglaenko

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

Graduate Department of Immunology
University of Toronto

© Copyright by Yuriy Baglaenko 2017
ABSTRACT

Dissecting the Genetics and Function of CD5⁺ B and invariant NKT cells using Congenic Lupus-Prone Mouse Strains.

Yuriy Baglaenko, Doctor of Philosophy Thesis, Department of Immunology, University of Toronto, 2017.

Systemic lupus erythematosus (SLE) is a complex multisystem autoimmune disorder characterized by the production of antibodies to nuclear antigens. Congenic strains derived from lupus-prone mice have been extremely useful in determining the pathogenesis of this condition. These mice, which harbor selected genetic elements containing susceptibility loci, can be used to identify key pathways and immune networks involved in disease initiation and progression. Alternatively, these same models can be used to identify the role of suppressive regulatory cells involved in preventing disease. In this thesis, the role and function of CD5⁺ and invariant Natural Killer T (iNKT) innate lymphocytes were investigated in the suppression of disease in New Zealand Black lupus-prone congenic mice. Through adoptive transfer and knockout experiments, CD5⁺ B cells, but not iNKT cells, were shown to suppress autoimmunity by inhibiting the frequencies of proinflammatory T cell subsets. As these CD5⁺ B cells secreted IL-10, which has been shown previously to suppress disease, the role of this cytokine was further investigated through knockout experiments that highlighted its critical role in maintaining CD5⁺ B, iNKT, and Natural Killer cell homeostasis. Finally, the immunogenetic basis of impaired iNKT cell function in the congenic mouse strains was
investigated, revealing a critical role for Ly108 in iNKT cell development and function. Collectively, these findings highlight the role of CD5^+ B cells and iNKT cells in SLE pathogenesis and demonstrate the power of congenic animals to dissect the genetic basis of disease.
DEDICATION

If you’ve ever had the onus, or rather privilege, of having dinner with my family, you’d have likely heard the following story:

When I was young, my mother took it upon herself to tutor both my brother and I in math. As she’ll tell you, I was a restless child and the only way that I would do a single math problem was upside down, quite literally standing on my head.

I can’t begin to imagine the kind of patience it must have taken to tutor a child with such unwillingness to learn. And so, I dedicate this thesis to Lina Otarova and Igor Baglaenko for inspiring a lifelong love of education. Thank you for supporting me, now and then.

All that I ever have and ever will accomplish is because of your unyielding love.
ACKNOWLEDGEMENTS

There is certainly a long list of people to thank, that have come in and out of my life during the last six years. I could not have done this alone, and although I can’t acknowledge you all individually, know that I am eternally grateful for your support. Still, I’d like to take a few pages and thank those that I can.

The PhD is a long journey that starts and ends with the supervisor and I am truly grateful to my mentor, Dr. Joan E Wither. Thank you for giving me the freedom and the support that I needed to grow as an independent researcher and chase some truly absurd ideas. Your passion for science and human health has been an inspiration throughout the years. Thank you for always being open to new experiments and ridiculous pet-projects; for listening and arguing; for editing and re-writing; and most importantly persevering. Through endless granting cycles, I’ve witnessed your fortitude and will always remember the resilience.

I must also acknowledge the unbridled support that I have received from my committee members, Dr. Michael Ratcliffe and Dr. Thierry Mallevaey. You’ve both gone above and far beyond what is expected. Mike, you introduced me to immunology and basic research, opening doors on my behalf. You’ve been a great supporter and a wonderful advisor. Thierry, you have been vital to my growth as a researcher and your expertise has been instrumental in my PhD. Thank you both for all that you’ve done for me. I know I’ve asked a lot of you over the last 6 years and I only hope that I can someday pay it forward.
The Wither lab with all its members, past and present, has been an incredible place to learn and grow. Thank you all. I need to especially thank my two mentors Dr. Christina Loh and Dr. Evelyn Pau. You’ve both been an inspiration and I truly value your guidance. With that, I must also acknowledge the endless support of Dr. Nan-Hua Chang and Kieran Manion; the Wither lab would not be the same without you. Kieran, I certainly don’t know what I would have done without our countless musical interludes. Finally, Kim Lifeso, I know we didn’t get along at times but somehow our friendship grew. Thanks for taking all my late night phone calls and pushing me to be much more honest with myself.

Graduate school has been a tremendously rewarding experience and I need to give my thanks to all the people that I’ve met throughout my various volunteer and leadership roles, including the extended IGSA family. It takes a community to raise a PhD. On that thread, I must acknowledgement the support that I’ve received from our department chair Dr. Zuniga-Pflucker. As always, thanks JC.

I know this will embarrass them both, but I need to thank my two brothers-in-science: Charles Tran and Eric Gracey. I’ve leaned heavily on both of you throughout the years, intellectually and emotionally. Charles, you and I have accomplished a lot together and I know we’ll do much more in the future. Eric, science at the Toronto Western (Krembil) would have been aimless without your input, advice, and various shenanigans. Sincerely, thank you both. There is no doubt in my mind that I would have quit my PhD years ago without the two of you.
I’ve personally found that working on multi-lab collaborative projects has been the most rewarding time of my PhD. Talking science and working towards a common goal is an incredible feeling. Therefore, I need to acknowledge the deep friendships that I’ve formed from that work, particularly with Robin Vigouroux and Dr. Nardos Tassew. Nardos, your dedication and calm demeanor is to be admired. Robin, your enthusiasm and optimism is unparalleled. I must also thank my other collaborators, Nick Toka, Ahmad Labban, and Mayra Cruz Tleugabulova for their support.

Science aside, I must once again thank the entirety of my extended family for their unconditional love. I want to specifically acknowledge my brother, Anton Baglaenko, and cousin, Yuri Otarov, for being lifelong confidantes and tireless advocates. If I’m ever down on my luck, you had better believe I’m calling you both. I must also take a beat to thank Sarah Chau and Melanie Zimmerman for their patience and support over the years. The PhD is a slog at times and I truly appreciated your friendships.

Finally, I must end by thanking April Pawluk. You’ve been an inspiration in this last year of my PhD and have pushed me to pursue my passions. Great things lie ahead for you and I only hope that I can be a part of them.
# TABLE OF CONTENTS

ABSTRACT .................................................................................................................................... ii  
DEDICATION ............................................................................................................................... iv  
ACKNOWLEDGEMENTS ............................................................................................................. v  
TABLE OF CONTENTS ............................................................................................................. viii  
LIST OF ABBREVIATIONS ........................................................................................................ xi  
LIST OF FIGURES ..................................................................................................................... xiv  
LIST OF PUBLICATIONS ........................................................................................................ xvii  
CHAPTER I INTRODUCTION ......................................................................................................1  

1 Systemic Lupus Erythematosus: the prototypic systemic autoimmune disorder ...............1  
   1.1 Patient Statistics, Manifestations, and Genetics ......................................................1  
   1.2 Immunopathogenesis of SLE .............................................................................4  
   1.3 Current Treatments ..........................................................................................6  
   1.4 Mouse models of SLE ..................................................................................8  
   1.4.1 Transgenic and Knockout Mouse models ..............................................8  
   1.4.2 Inducible Mouse Models .......................................................................9  
   1.4.3 Spontaneous mouse models of disease and related congenic mice ......11  
2 The critical role of B and T cell interactions in SLE ......................................................17  
   2.1 B cell development and implications in autoimmunity ....................................17  
   2.2 T cell help, germinal centers, and inflammatory T cell subsets ....................20  
3 Ontogeny, function, and role of innate B1 cells in SLE ..................................................23  
   3.1 B1 cell development .....................................................................................24  
   3.2 B1 cell functions ..........................................................................................26
Discussion ..........................................................................................................................90

CHAPTER III  IL-10 Production is Critical for Sustaining the Expansion of CD5+ B and NKT cells and Restraining Autoantibody Production in Congenic Lupus-Prone Mice. ...............................................................................................................................95

Abstract ..................................................................................................................................96

Introduction ............................................................................................................................97

Materials and Methods .......................................................................................................101

Results .....................................................................................................................................104

Discussion ..............................................................................................................................123

CHAPTER IV  iNKT cell activation is critically dependent on homotypic trans-Ly108 interactions..............................................................................................................................128

Abstract ..................................................................................................................................129

Introduction ............................................................................................................................130

Materials and Methods .......................................................................................................134

Results .....................................................................................................................................141

Discussion ..............................................................................................................................166

CHAPTER V  DISCUSSION AND FUTURE DIRECTIONS ...................................................173

REFERENCES ............................................................................................................................181

COPYRIGHT ACKNOWLEDGEMENTS .................................................................................212
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANA</td>
<td>Anti-nuclear antibody</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>B</td>
<td>B Lymphocyte</td>
</tr>
<tr>
<td>B1</td>
<td>B1 Lymphocyte</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Breg</td>
<td>Regulatory B</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>Flt3L</td>
<td>FMS-like tyrosine kinase-3</td>
</tr>
<tr>
<td>FO</td>
<td>Follicular</td>
</tr>
<tr>
<td>GN</td>
<td>Glomerulonephritis</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association study</td>
</tr>
<tr>
<td>HEL</td>
<td>Hen egg white lysozyme</td>
</tr>
<tr>
<td>hi</td>
<td>High</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IFNα</td>
<td>Interferon alpha</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILC</td>
<td>Innate lymphoid cell</td>
</tr>
<tr>
<td>iNKT</td>
<td>Invariant Natural Killer T cell</td>
</tr>
<tr>
<td>int</td>
<td>Intermediate</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>LM</td>
<td>Light microscopy</td>
</tr>
<tr>
<td>lo</td>
<td>Low</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>MZ</td>
<td>Marginal zone</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>NZB</td>
<td>New Zealand Black</td>
</tr>
<tr>
<td>NZB/W F1</td>
<td>New Zealand Black x New Zealand White F1</td>
</tr>
<tr>
<td>NZM</td>
<td>New Zealand Mixed</td>
</tr>
<tr>
<td>NZW</td>
<td>New Zealand White</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>SAP</td>
<td>SLAM associated protein</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency disorder</td>
</tr>
</tbody>
</table>
SLAM  Signaling lymphocyte activation molecule
SLE   Systemic lupus erythematosus
SNP   Single nucleotide polymorphism
ssDNA Single stranded DNA
T      T Lymphocyte
TCR   T cell receptor
Tfh   T follicular helper
TGF   Transforming growth factor
Th1   T helper 1
Th17  T helper 17
Th2   T helper 2
TLR   Toll-like receptor
TMPD  2,6,10,14-tetramethylpentadecane
TNFα  Tumour necrosis factor alpha
Treg  Regulatory T
αGalcer α-Galactosylceramide
LIST OF FIGURES

Figure 1: The cycle of SLE pathogenesis and intervention strategies ............................................ 7

Figure 2: iNKT cell development diverges into three distinct subsets as identified by cell-surface markers and transcriptional factors ........................................................................ 41

Figure 3: Genomic organization of SLAM receptors .................................................................. 47

Figure 4: Mouse and human SLAM receptors ......................................................................... 48

Figure 5: Knockout of NKT cells has no effect on the suppression of fatal autoimmunity in B6.NZBc1c4 mice. ....................................................................................................... 70

Figure 6: Knockout of NKT cells has no impact on the expansion of CD5+B cells or IL-10-producing B cells. .................................................................................................................. 71

Figure 7: B6.NZBc4 and suppressed B6.NZBc1c4 mice have an expansion of IL-10-producing CD5+B cells. ...................................................................................................................... 74

Figure 8: Phenotypic characterization of IL-10 producing B cells using typical B1a cell markers ................................................................................................................................. 77

Figure 9: IL-10 producing plasmablasts are expanded in suppressed mice but represent a rare population in the IL-10+B cell pool. .................................................................................. 78

Figure 10: CD5+B cells can respond to stimulation with nuclear autoantigens and TLR ligands. ................................................................................................................................. 80

Figure 11: The expansion of pro-inflammatory T cell subsets is reduced in B6.NZBc1c4 mice and correlates with an increase in IL-10-producing B cells ....................................................... 82

Figure 12: Lack of correlation between T regulatory cells and the expansion of CD5+B cells in B6.NZBc1c4 mice. .............................................................................................................. 84

Figure 13: Adoptively transferred B cells from B6 and B6.NZBc4 mice can be identified in host autoimmune animals but do not express CD5 ........................................................................ 87

Figure 14: Transfer of total B cells from B6.NZBc4 mice reduces the frequencies of pro-inflammatory IL-17A- and IFN-γ-producing CD4 T cells but has no impact on autoantibody levels. .................................................................................. 89

Figure 15: The expansion of peritoneal B1a B cells, splenic CD5+B cells, and NKT cells localizes to an NZB-derived interval spanning 91 to 123 Mb on chromosome 4. ............ 106
Figure 16: Total spleen and peritoneal cavity counts. .................................................... 107

Figure 17: Suppression of anti-ssDNA and –dsDNA autoantibody production in bicongenic B6.NZBc1c4(123-151) mice in the absence of either CD5+ B cells or splenic NKT cell expansion. ........................................................................................................ 109

Figure 18: Knockout of IL-10 is penetrant in B6, B6.NZBc4m, and B6.NZBc4 mice. 111

Figure 19: Knockout of IL-10 in B6.NZBc4 but not B6.NZBc4m mice results in a breach of tolerance to ssDNA, dsDNA, and chromatin. ............................................................ 112

Figure 20: Levels of IgG1 and IgG2a autoantibodies in IL-10 knockout mice are unchanged. ...................................................................................................................... 113

Figure 21: The expansion of CD5+ B cells, NK and NKT cells is impacted by the loss of IL-10. .............................................................................................................................. 117

Figure 22: The frequency of splenic regulatory T cells is unchanged in B6.NZBc4m IL-10 knockout mice. ........................................................................................................... 118

Figure 23: Knockout of IL-10 in full-length B6.NZBc4 but not B6.NZBc4m mice resulted in a loss of transitional B cells and an expansion of marginal zone B cells..... 119

Figure 24: IL-10 is required for cell survival but has no impact on the proliferation of peritoneal B1a cells ........................................................................................................ 122

Figure 25: Reduced splenic iNKT cell frequency and skewed peripheral subsets in B6.NZB(171-177) mice. .................................................................................................................... 142

Figure 26: Reduced thymic iNKT cell frequencies and alteration in Ly108, CD150, and Ly9 expression in B6.NZB(171-177) mice. .............................................................................. 145

Figure 27: B6.NZB(171-177) mice have skewed iNKT cell development and altered NKT cells subsets. ........................................................................................................ 148

Figure 28: iNKT cells from B6.NZB(171-177) mice are functionally defective and have deficiencies in SLAM family expression .............................................................. 151

Figure 29: Only injection of B6.NZB(171-177) BMDCs into B6.NZB(171-177) recipients can recapitulate the loss of IFNγ and IL-4 production in iNKT cells. .............. 153

Figure 30: B6.NZB(171-177) BMDCs have decreased Ly108 expression but no changes in αGalCer presentation or conjugate formation with NKT cells. .................... 155

Figure 31: Silencing of Ly108 or inhibition of trans-Ly108 signaling reduces NKT cell activation ................................................................. 158
Figure 32: Loss of Ly108 leads to ineffective PLZF and Erg2 upregulation in pre-selection DP Thymocytes. ................................................................. 161

Figure 33: Ly108 signaling in human iNKT cells augments activation. ......................... 165
LIST OF PUBLICATIONS

1. **Baglaenko Y**, Cruz Tleugabulova M, Gracey E, Talaei N, Manion KP, Chang N, Mallevaey T & Wither JE. iNKT cell activation is critically dependent on homotypic trans-Ly108 interactions. [Manuscript submitted]


CHAPTER I
INTRODUCTION

1 Systemic Lupus Erythematosus: the prototypic systemic autoimmune disorder

Systemic Lupus Erythematosus (SLE) is an autoimmune disorder characterized by the production of anti-nuclear autoantibodies that deposit in the tissues, causing damage, and leading to significant morbidity and mortality. Unlike Sjogren’s syndrome or dermatomyositis, two other systemic autoimmune disorders, SLE can affect virtually every organ of the body. Consequently, SLE is often described as the prototypic systemic autoimmune disorder [1].

1.1 Patient Statistics, Manifestations, and Genetics

With an ever-increasing prevalence of 52.2 in 100 000 in the US, SLE disproportionately affects women in their child-bearing years [1]. Although females are most likely to be affected (at a ratio of 9 to 1), longitudinal studies have not identified differences in clinical severity or outcome based on sex [2]. SLE is a clinically diverse disorder whose diagnosis relies on meeting 4 of 11 American College of Rheumatology criteria that include the presence of autoantibodies, hematologic changes, and clinical manifestations.

SLE is a complex autoimmune disease that is initiated and exacerbated through the interaction of genetics and the environment. The relative contribution of each factor
to pathogenesis is difficult to discern, but from mono- and di-zygotic twin studies, the presence of a genetic component to SLE is well established. Familial studies also support a strong genetic component to SLE initiation, progression, and pathogenesis with first-degree relatives showing a >20 fold increase in risk [3]. Clearly, genetic variation can impact on SLE susceptibility.

From years of work, we can discern that genetic differences fall into three broad categories: rare (<1%) genetic polymorphisms and copy number variants; common (>1%) single-nucleotide polymorphisms (SNPs) and copy number variants; and epigenetic modifications [3]. Recent initiatives in genome wide association studies have helped to identify common variants (SNPs) that might additively support the course of disease. The strongest association, as with most autoimmune disorders, is the MHC locus representing polymorphisms in both classical MHC I and II, and non-classical class III molecules. In SLE, HLA-DR3 has been shown to be associated with increased anti-DNA autoantibody production [4]. A recent GWAS study verified and identified a number of polymorphisms in key adaptive and innate immune genes that may contribute to SLE pathogenesis [5]. To date, 43 susceptibility loci have been identified with 16 SLE-linked transcription factors. This is in line with the hypothesis that dysregulated genomic networks underpin lupus pathogenesis. Of particular interest, a SNP in IL-10 has repeatedly been identified to be associated with SLE [4,5]. However, as these studies rely on association of quantitative trait loci, the exact causal gene is difficult to discern. In fact, in some cases, numerous plausible genes can be found in close proximity to the
associated loci. Future work will certainly require the validation of SLE-associated genes and a better understanding of the networks of responses in different cell types and under different inflammatory conditions. Furthermore, SNPs identified by GWAS studies still cannot account for all the variation in SLE incidence, and it is therefore highly likely that other genetic variations and mechanisms, as outlined above, are also involved in the pathogenesis of SLE.

Besides genetic factors, environmental factors such as sunlight, cigarette smoking, infection, vaccination, chemical exposure, vitamin D deficiency, exogenous estrogen, and drug use can impact on SLE progression [1,3]. The most well-recognized environmental factor is sunlight exposure, which can exacerbate the underlying typical lupus skin rash by causing apoptosis and consequent autoantigen release [6,7]. A recent addition to this list is anti-TNFα treatment, which is a commonly prescribed biologic for rheumatoid arthritis and Crohn’s disease. These therapies are frequently associated with development of a positive ANA test and in a minor subset of patients, result in the onset of lupus-like symptoms, primarily skin rashes [8].

Taken together, these findings indicate that SLE has a complex and multifaceted pathogenesis. Although there is a clear underlying genetic component to SLE, environment factors contribute to promote disease. The current concept of disease pathogenesis suggests that many minor but additive abnormalities in critical immune genes, identified through GWAS studies, combine to create susceptible individuals which
are prone to SLE. Environmental cues can then contribute to push these susceptible individuals towards clinical autoimmunity.

1.2 Immunopathogenesis of SLE

In predisposed individuals, the compounding cycle of SLE is initiated by concurrent breaches of tolerance in T and B cells and an abundance of autoantigens erroneously presented by antigen-presenting cells (APCs). Broadly speaking, breakdowns in checkpoints that control both the innate and adaptive immune response combine to promote autoimmunity.

The innate immune system comprises both the physical and mucosal barriers as well the collection of innate immune cells, including granulocytes, monocytes, and dendritic cells (DCs). Under normal conditions, activation of these cells is limited and spatially restricted. However, in the case of autoimmunity, defects in a number of pathways lead to incorrect and exacerbated activation. The resultant production of pro-inflammatory cytokines and chemokines further drives immune activation. Numerous studies have implicated perturbed DC function and activation in SLE pathogenesis [9]. The direct processing and presentation of autoantigens to B and T cells or the indirect production of IL-12 and IFNα can contribute to driving a pro-inflammatory autoimmune response [10]. IFNα production by plasmacytoid dendritic cells, in particular, has recently been shown to play a central role in disease progression [10–15]. Genes associated with IFNα responses such as IRF5, IRF7, and TYK2 continue to be identified in association studies, strengthening their relative importance to disease pathogenesis in
affected individuals [16,17]. In DCs, many of these abnormalities are linked to defective TLR signaling to nuclear antigens and immune complexes which results in hyperactivation and presentation of autoimmune antigens.

The corresponding adaptive immune response, which is activated by antigen presenting and innate cells, continues the cycle of autoimmunity and drives pathogenesis. Unlike the innate immune response, the adaptive arm, comprised of B and T cells, is educated in the bone marrow and thymus, respectively. This process acts to remove self-reactive cells through central tolerance checkpoints in these organs. Peripheral tolerance through the action of regulatory cells or the absence of co-stimulation further restrains unwanted immune responses. In SLE, these mechanisms and checkpoints, both centrally and peripherally, are disturbed [18]. The resultant activation of autoantigen specific B and T cells leads to the differentiation of plasma cells and autoantibody production. Autoantibodies can bind directly to target tissues disturbing function and/or form immune complexes with nuclear antigens that deposit in target tissues and drive damage, leading to morbidity. Additionally, the formation of immune complexes activates the innate immune system and continues the cycle of autoimmunity. In this way, the positive feedback of autoimmunity is established as activation of immune cells continues to drive pathogenesis, unless restricted [18,19].

The final component of SLE pathogenesis is the release of autoantigens in target tissues and lymphoid organs. Normal homeostatic turnover of cells and tissues is tightly
regulated in healthy individuals. Patients with SLE, however, have defects in pathways that increase necrotic cell death, lead to improper clearance of apoptotic debris, or as recently implicated, increase neutrophil NETosis [20]. In each of these cases, the resultant abundance of nuclear antigen is presented by activated immune cells and the cycle of immunopathogenesis is exacerbated.

Of course, the end result of this entire process is direct tissue damage caused by immune cell activation, which in the case of SLE can impact on any organ but is frequently manifested by skin rashes and kidney damage. Tissue damage can occur by a number of mechanisms and is not always correlated to direct immune infiltration. In cases of SLE nephritis, deposition of complement and immune complexes on glomeruli are key indicators of disease. Soluble inflammatory mediators and cell infiltrates can also cause tissue damage. Additionally, genes that impact on renal cell regeneration and fibrosis have also been linked to SLE pathogenesis [21].

1.3 Current Treatments
SLE is a complex disease, both mechanistically and pathogenically, making it decidedly less treatable than other diseases such as arthritis. Current frontline treatments primarily promote immunosuppression through the use of drugs such as corticosteroids, chloroquines, and cytotoxic agents such as Imuran, Cellcept, and Cyclophosphamide. Patients unresponsive to these conventional interventions may receive targeted biologic therapies, many of which are still undergoing clinical trials, aimed at blocking critical cytokines, survival factors, and B and T cells (Figure 1). These treatments include: B cell
depleting antibodies such as rituximab (anti-CD20) and epratuzumab (anti-CD22); T cell

Figure 1: The cycle of SLE pathogenesis and intervention strategies

Figure outlining the immune cycle of pathogenesis in SLE and possible therapeutic targets. Reproduced with permission from [22]. Of the potential targets listed, only anti-BAFF, denoted with a *, has been approved for treatment of SLE. Many others are still in ongoing clinical trials.
targeting therapeutics such as abatacept (CTLA4-Fc fusion protein) and ruplizumab (anti-CD40L); and anti-cytokine treatments such as sifalimumab (anti-IFNα) and belimumab (anti-BAFF/BLyS) [22]. Combined, these multi-billion dollar treatments represent a new subset of therapeutics which have shown promising efficacy in a number of autoimmune disorders, although with some notable side effects. All of these treatments arose from a fundamental understanding of the mechanisms involved in pathogenesis and rely on the use of animal models to decipher the intricacies of the SLE-promoting genes, cell types, and pathways.

1.4 Mouse models of SLE
Several mouse models have been developed that closely mimic the pathology and/or pathogenesis of SLE. These genetically manipulated (transgenic or knockout), inducible, and congenic models have been critical in helping to decipher the genetics of this complex disease and to develop therapeutics. Spontaneous congenic models have been particularly useful in unraveling the genetic abnormalities that support lupus pathogenesis and are the major focus of the presented thesis.

1.4.1 Transgenic and Knockout Mouse models
Since SLE is a polygenic disorder, transgenic and knockout mouse models are not reflective of the genetic complexity in SLE. Nevertheless, these mice have revealed critical roles for key genes involved in the pathogenesis of disease.
C57BL/6 animals, although skewed towards a predominantly Th1 phenotype, do not develop autoimmunity. However, these animals are permissive to disease and numerous studies have identified knockouts that result in SLE-like autoimmunity characterized by autoantibody production and glomerulonephritis [23]. Typically, knockout animals that mimic disease fall into two categories: A) those that impact on apoptosis and apoptotic debris clearance or B) checkpoint proteins in B and T cell activation and proliferation. Knockout of Serum amyloid protein, DNAse I, and C1q all fall into the first category of proteins and result in autoimmunity by presumably creating an excess of autoantigens [24–26]. C1q deficiency has specifically been linked with SLE in patients [27,28]. Knockouts in the second group include Lyn, Fyn, CTLA-4, and PD-1, to name a few, which together with transgenic overexpression of molecules such as Blys and Bcl2 [29–34] modulate signaling, leading to over-activation and/or improper selection T and/or B cells, causing erroneous activation of autoreactive lymphocytes. As before, some of these critical genes have been validated in patients, including PD-1 and Lyn [35,36]. Interestingly, many of these genes, including CTLA-4, C1q, PD-1, and Lyn, are located on mouse chromosome 1 and 4.

1.4.2 Inducible Mouse Models
As an alternative approach, the underlying mechanisms of SLE pathogenesis can be partially recapitulated in induced models of disease. These models have the advantage of allowing the study of genetic elements that would otherwise not cause disease, within a permissive environment.
Pristane (2,6,10,14-tetramethylpentadecane) or TMPD, is a naturally-occurring hydrocarbon oil that when injected into the peritoneal cavity can be used to drive chronic inflammation [37]. The lupus-like symptoms that arise from its use have been linked with the overproduction of type I interferons, including IFNα and IFNβ. Administration of this compound to BALB/c mice leads to an RA and lupus-like disease with production of autoantibodies including anti-dsDNA, -ssDNA, -chromatin, -Sm, -RNP, -Su, and – ribosomal P [38–40]. Besides type I IFNs, IL-6, IFNγ, and IL-12 are all critical to sustaining and driving autoimmunity. Highlighting the critical role of IFN in this model, IFNAR deficient mice are resistant to production of IgG anti-chromatin and anti-DNA antibodies and develop less severe glomerulonephritis [41]. TLR7 deficient mice have abolished autoantibody production and nephritis and induction of disease is possible in germ-free mice, suggesting that pristane administration creates endogenous TLR7 ligands possibly through accumulation of apoptotic debris, mimicking human disease [42]. Interestingly, not all inbred strains are equally susceptible to disease. C57BL/6 mice develop a milder form of disease when compared to SJL or BALB/c animals, implicating a role for other genes in disease susceptibility [37].

Several lines of evidence suggest that an IFN signature and signaling is critical to SLE pathogenesis. As an alternative to pristane administration, IFN overexpression can be induced by adenoviral vectors. These studies allow for the upregulation of IFN in the absence of the other overt phenotypes caused by pristane administration. Again, IFN upregulation can induce SLE but only in autoimmune prone mice through the induction
of IL-6 and BAFF [43,44]. These models, which rely on the overproduction of type I IFNs, may mimic the IFN signature found in SLE patients and are therefore potentially useful for the testing of approaches that target this pathway.

Another well-established inducible model of autoimmunity is chronic graft-versus-host disease (cGVHD) wherein alloreactive donor cells are injected into hosts. In one variation of cGVHD, C57BL/6 splenocytes are injected into C57Bl/6-H2b^{bm12} recipients (that harbor a three amino acid substitution in H-2b), or vice versa, causing the production of autoantibodies such as anti-dsDNA and -chromatin through the allore cognition of transferred T and host B cells. In this model, autoimmunity develops robustly within 10-14 days and is largely dependent on strong T cell help that leads to a substantial breach in B cell tolerance [45].

1.4.3 Spontaneous mouse models of disease and related congenic mice
Although studies with transgenic and inducible animals allow for a better understanding of the core mechanisms of disease, their relevance to patients remains limited. Except for the few rare cases, most patients have less dramatic functional abnormalities arising from numerous genetic loci all of which combine to promote lupus. In this regard, spontaneous models allow for the study of disease initiation and progression in a more applicable setting. Furthermore, genetically susceptible animals that spontaneously develop disease allow for the discovery and mapping of critical lupus-promoting or suppressing genes.
One well-characterized spontaneous model of lupus-like autoimmunity is the MRL/lpr mouse, which has a loss-of-function mutation in the Fas gene. This mouse model was created serendipitously by a mutation in the MRL inbred line, an autoimmune-prone strain in its own right. Disease in these MRL/lpr animals, which has a 50% mortality rate and a female bias, is characterized by splenomegaly and abnormal T and B cell activation [46]. Although the Fas mutation likely drives autoimmunity, studies with a similar mutation in non-autoimmune prone C57Bl/6 mice revealed that this defect alone was not sufficient to reproduce severe autoimmunity [47]. This finding indicates, once again, that SLE is a polygenic disorder.

1.4.3.1  NZB/W F1, NZM2410, and Sle1-3 mouse strains of autoimmunity
The classic spontaneous mouse model of SLE is the New Zealand Black (NZB) and New Zealand White (NZW) F1 (NZB/W F1) cross which develops autoantibodies and renal pathology by 10-12 months of age that culminates in mortality. Unlike humans with SLE, NZB/W F1 mice do not develop anti-RNA autoantibodies. However, similar to SLE patients, there is a strong female bias with production of anti-chromatin, -ssDNA, and –ds DNA antibodies leading to renal disease. Notably, ovariectomy can delay onset of disease and reduce ANA titres, suggesting a role for estrogens in disease pathogenesis [48]. NZB/W F1 mice are a well-studied and reliable model of disease that has been used to test a number of effective SLE therapeutics.
It is interesting to note that neither parental strain develops fatal autoimmunity. NZB mice have polyclonal B and T cell activation with anti-ssDNA autoantibodies while NZW develop late stage nephritis [49,50]. It is only the F1 cross that facilitates the progression of fatal autoimmunity. In an effort to find genetic loci related to pathogenesis, subsequent studies have focused on generating congenic mice created through successive breeding and backcrossing strategies. Traditionally, congenic mice are generated by breeding autoimmune prone mice onto a parental non-autoimmune background, or vice versa. F1 progeny are then backcrossed onto the genetic background of choice and random recombination events along the chromosomes create a patchwork of loci that can be used to generate smaller introgressed intervals. At the final stage, brother sister breeding culminates in fully inbred lines.

For NZB/W F1 mice, the first derivative congenic lines were generated serendipitously. Originally bred for coat color, an NZB/W F1 female was backcrossed with an NZW male which led to a mismatch of various colors. The resultant progeny was continually mated as brother-sister pairs until 27 fully inbred New Zealand Mixed (NZM) lines were established of varying lupus penetrance. One of the derived strains, NZM2410 (tan in colour), had a high penetrance of disease with 80% of mice developing nephritis by 12 months of age but a surprisingly weaker female bias. Overall, these mice have roughly 75% NZW- and 25% NZB-derived DNA. However, the key benefit of homozygosity allowed for a more detailed understanding of the genetics of disease [51–53].
Investigation of the genetics of SLE pathogenesis in NZM2410 mice began with a linkage analysis to identify relevant loci. In these studies, NZM2410 mice were initially crossed with non-autoimmune B6 mice. The F1 progeny were then backcrossed onto the NZM2410 background and the progeny screened for key SLE phenotypes. Three NZM-derived loci linked to glomerulonephritis were identified: Sle1 located on mouse chromosome 1, Sle2 on mouse chromosome 4, and Sle3 on mouse chromosome 7 [53]. The importance of these three loci and chromosomal intervals in the pathogenesis of murine lupus was confirmed by additional mapping studies in other mouse strains including NZB, MRL, and SWR [54–57].

Each of these intervals was further investigated through generation of congenic mice, bred onto the non-autoimmune B6 background. The resultant strains were entirely B6 except for introgressed intervals containing the NZM2410 lupus-susceptibility loci. Although each Sle interval could independently promote lupus-associated phenotypes, only the combination of all three intervals could recapitulate fatal autoimmunity [58]. Sle1 was noted to increased anti-chromatin IgG and promote B and T cell activation [59,60]. This interval, which was later discovered to be entirely NZW-derived contains a number of potential lupus-promoting genes including the SLAM family of molecules (notably Ly108 and CD84), CR2, and FcγR2b, all of which have been shown to contribute to autoimmunity. Recently, another lupus susceptibility gene, Pbx1, was identified on mouse chromosome 1 within the Sle1 interval and shown to promote the generation of T follicular helper cells, at the expense of regulatory T cells [61]. Sle2 has
been shown to lower the threshold of B cell activation and promote expansion of B1a cells in the absence of autoimmunity [62,63]. Potential lupus-promoting genes within this interval include p18, IFNα, TLR4, and FAAH [64]. Finally, Sle3 has been shown to promote CD4 T cell activation mediated by a myeloid cell defect [60,65,66]. This interval includes the kallikrein genes which play a renoprotective role with polymorphisms in KLK3 identified in SLE patients [51,52,67]. Studies with these mice revealed that several distinct immune abnormalities arising from independent genetic loci could promote SLE-pathogenesis and interact with each other to promote fatal disease.

1.4.3.2 NZB-derived congenic mouse strains

Apart from the distal region of Sle2, the entirety of the identified lupus-promoting regions from NZM2410 animals belongs to the NZW background. Yet, linkage studies have shown that there are lupus promoting genes on NZB mouse chromosome 1, 4, 7, and 13 [52]. Additionally, NZB and NZW mice are highly polymorphic, with close to 15% of identified SNPs varying between these two strains. To put this in perspective, NZB and B6 mice are roughly equivalently polymorphic [52]. Thus, it is likely that many of the lupus susceptibility genes in these two strains are distinct.

Work from our laboratory has focused on the generation of chromosome 1, 4, and 13 congenic animals derived solely from the NZB background. Similar to previous findings with NZM2410 derived animals, we have identified numerous autoimmune susceptibility genes on mouse chromosomes 1 and 13. Furthermore, within the NZB chromosome intervals, there are several distinct SLE-promoting loci. The creation of
subcongenic mice, with shorter NZB-derived intervals, can identify the various intervals and associated defects. Using these chromosome 1 subcongenic animals we have shown that there are at least 3 lupus susceptibility genes between 125-177Mb [68]. Together, these defects combine to enhance the generation of pathogenic Th1, Th17 and follicular helper T cells which promote autoimmunity [68]. A follow-up study on these intervals identified a defect in EAT-2, an adaptor protein for SLAM signaling. It was shown that a polymorphism in the EAT-2 gene leads to decreased expression of this negative signaling molecule on DCs and thereby enhances cytokine production [69]. For NZB chromosome 13 mice, at least two independent loci appeared to promote autoimmunity. One locus was associated with B cell hyperresponsiveness to TLR3 stimulation, whereas the other led to impaired clearance of apoptotic debris by macrophages [70,71]. Work on NZB-derived mouse chromosome 4 congenics, has revealed that like Sle2, there is an expansion of B1a cells in the absence of overt autoimmunity. However, highlighting the differences between the NZB and NZM mouse strains due to their differences in genetic background, the NZB chromosome 4 congenic mice generated in our laboratory had an expansion of both splenic and hepatic NKT cells [72]. Interestingly, when triple congenic mice were created with Sle1, Sle2, and Sle3, the presence of the Sle2 locus greatly exacerbated disease progression and increased mortality [73]. However, in our NZB derived animals, the creation of bicongenic mice with both chromosome 1 and 4 intervals resulted in suppressed autoimmunity with increased survival, reduced renal disease and a bias towards less-pathogenic IgG1 ANAs [74]. Notably, these bicongenic mice retained expansion of iNKT and B1a cells, two innate immune cell populations that have been
proposed to regulate autoimmunity. This finding suggested that one or both of these populations could lead to suppression of disease in bicongenic mice, a possibility that is further explored in this thesis.

2 The critical role of B and T cell interactions in SLE

B cells are central players in the proper orchestration of the adaptive immune response. Their functions are wide-ranging and include antibody secretion, antigen presentation, and cytokine production. As precursors to memory B cells, long-lived plasma cells, and short-lived plasmablasts, B cells are generally thought of as drivers of autoimmunity in SLE. In this regard, defects in their selection, survival, activation, and differentiation have all been associated with SLE in multiple mouse models. However, for the generation of long-lived high-avidity autoantibody responses, hallmarks of pathogenic autoimmunity, B cells require T cell help. Helper T cells, which must simultaneously recognize autoantigens, further support and drive the immune response by enhancing germinal center responses. Thus, defects in T cell selection and activation are equally culpable in promoting autoimmunity and are also highly associated with SLE [75].

2.1 B cell development and implications in autoimmunity

B cells develop from common lymphoid progenitors in the bone marrow. Commitment to the B cell lineage is associated with an order rearrangement of heavy (H) and light (L) chain Ig loci resulting in the acquisition of B cell receptors (BCR) through a stochastic process of VDJ recombination. The need to create a wide repertoire of antigen-specific B cells coupled with the inherently random nature of this process creates a surprisingly high
proportion of autoreactive B cells with upwards to 75% of the immature compartment recognizing self-antigens [76]. Fortunately, autoreactivity is kept in check by central tolerance mechanisms including BCR editing, clonal deletion, and anergy induction.

The first major checkpoint in B cell development is the formation of the preBCR complex wherein a surrogate L chain, comprised of VpreB and λ5, pairs with a recombined heavy chain. Successful pairing creates a basal signal through the preBCR complex which is critical to progression of B cell development [77,78]. This acts as a positive selection checkpoint and may be dependent on binding to self-antigens including heparin sulfate and galactin-1 [79,80]. Interestingly, this step may also act as a negative selection process. In studies of animals with knockout of surrogate light chains, there was a noted breach in tolerance leading to the production of autoantibodies [81]. This may be because the only B cells which can overcome this checkpoint are those with very strong Ig H chain signaling capacity, potentially enriching for autoreactivity.

Successful pairing of an H chain with the surrogate L chains terminates the pro-B cell stage and triggers the beginning of L chain rearrangements. While successful rearrangement of an L chain is required for further B cell development, this also leads to another important negative selection checkpoint. Receptor engagement with self-antigen at the pre-B cell stage initiates receptor editing, a process in which proximal L chain V and distal J segments (or alternative L chain loci) are recombined [82]. This process removes the large majority of autoreactive clones, as 25% of peripheral B cells have
edited L chain loci, suggesting some form of receptor editing [75,83]. Importantly, in mouse models of lupus and SLE patients, receptor editing is often impaired with lower frequencies of edited L chains observed in the peripheral B cell pool [84,85]. In the autoimmune prone Sle1 or Sle2 mice, for instance, it has been shown that defects in receptor editing can contribute to disease by preventing BCR revision [86,87].

B cells that escape the bone marrow enter the circulation as immature transitional B cells and migrate to secondary lymphoid organs as transitional B cells. Maturation and entry of transitional B cells into the various mature B cell compartments requires a functional Ig receptor, with the strength of the signal dictating which compartment the B cell enters [88,89]. Marginal zone (MZ) B cells are typically self-renewing cells that reside in specialized compartments close to vasculature and respond rapidly to blood-borne antigens in a largely T cell independent response [90]. Follicular (FO) B cells represent the majority of mature B cells and respond to both T cell dependent and independent antigens. For transitional B cells, the primary mechanisms for control against autoimmunity are clonal deletion and induction of anergy; an unresponsive state characterized by constant antigen engagement of the BCR [75]. These two mechanisms are not mutually exclusive, as cells that are rendered anergic at the transitional B cell stage are often purged prior to entry into the mature B cell compartment. This is because survival of mature B cells is dependent upon the B cell survival factor, BAFF, and anergic cells are refractory to BAFF signaling. Anergy in B cells may be maintained through recruitment and phosphorylation of phosphatases such as SHP-1 and SHIP-1.
The mechanisms leading to activation of these molecules may vary from cell to cell, and may include upregulation of PTEN or monophosphorylation of Igα and Igβ [91,92]. Despite the purging of many anergic B cells, anergic B cells can still be identified within the mature B cell repertoire of healthy individuals and are enriched for self-reactive cells [93]. SLE patients have increased proportions of autoreactive B cells in their mature B cell compartment and there is evidence that these cells may be less refractory to activation than those seen in healthy controls. These findings point to the idea that anergy may be overcome or reversible in SLE, and work on several transgenic/knockout mouse models of lupus has shown that factors that promote a breach in anergy also promote lupus-like disease. One way that anergy can be overcome is through concurrent BCR and Toll-like receptor (TLR) signals. Notably, GWAS studies have identified lupus risk variants in IRAK1, IRF5, and A20, all of which lie downstream of or modify TLR signaling [94–97]. Although TLR signaling can promote breaches of anergy and lead to expansions of autoantibody secreting plasmablasts, the generation of highly pathogenic ANAs is dependent on germinal center B cell reactions and T cell help.

2.2 T cell help, germinal centers, and inflammatory T cell subsets

Generation of high affinity antibodies is dependent on somatic hypermutation, a process which induces mutations in the VDJ regions of the BCR and is primarily driven by costimulatory factors from T cells. The process of somatic hypermutation generally occurs within germinal centers of secondary lymphoid organs. Germinal centers are sites of B cell proliferation and competition, wherein mutated B cell clones compete for antigen and T cell help. The most efficient BCRs can uptake the most antigen and receive
the necessary help to drive differentiation into memory B cells or plasma cells. The importance of this process to development of SLE is highlighted by the presence of spontaneous germinal centers in most murine models. Interestingly, it was recently discovered that BCR signaling is shut down during germinal center responses due to high phosphatase activity [98]. This is thought to promote antigen uptake and presentation to T cells signifying the critical role that helper T cells play in this reaction.

T follicular helper (Tfh) cells are critical to the generation of high-affinity antibodies and memory B cell responses. Identified by the expression of the transcription factor Bcl6 as well as cell surface CXCR5, PD-1, and ICOS, these cells localize within B cell follicles and support germinal center reactions [99]. Secretion of IL-21 by Tfh cells supports the differentiation and class-switching of B cells [100]. In NZB and other mouse models of SLE, increased frequencies of Tfh cells are shown to be associated with disease [68]. The generation of Tfh cells begins with the priming of naive T cells by dendritic cells. Like the other T helper subtypes, the strength of signal and differences in secondary signals drive the generation of these cells. For Tfh cells, IL-6, IL-21, and ICOS signaling is critical to differentiation and induces the expression of Bcl6, the master transcriptional regulator [101–103].

The other three main types of helper T cells include IFNγ-producing Th1, IL-17-producing Th17, and IL4-producing Th2 cells. Like Tfh cells, these other helper subtypes differentiate from a naive CD4 T cell precursor and are supported by expression of
critical transcription factors. Th1 cell differentiation is supported by expression of Tbet and stimulation by IFNγ and IL-12. Th2 cells differentiate in response to TCR stimulation and IL-4 signaling and require expression of GATA3 for expression of IL-4, IL-5, and IL-13. Finally, Th17 cells, which differentiate in response to IL-6 and TGFβ, require the expression of RORγT for transcription of IL-17 [104]. The expansion / skewing of these helper T cell subsets has been implicated in a number of autoimmune diseases, including SLE [105,106]. Through the production of inflammatory cytokines, these T helper subsets can further exacerbate the immune response and directly cause damage in tissues. The abundant IFNγ produced by Th1 cells, for instance, has been associated with disease severity in SLE patients [107].

With the discovery of Th17 cells, numerous studies have implicated their function in lupus pathogenesis. IL-17 levels are elevated in patients and in some cases correlated with disease activity [108–110]. In particular, lupus nephritis patients were shown to have high levels of IL-17 [111]. Since IL-17 can be produced by a number of immune cell populations, studies have also confirmed that the frequency of Th17 cells is increased in the peripheral blood of SLE patients, providing evidence for a direct link to disease [112,113]. The exact role of IL-17 in driving lupus pathogenesis has yet to be elucidated. However, murine studies have revealed that Th17 cells can infiltrate the kidney and that their ablation with an IL-23R knockout can prevent nephritis [114]. Germinal center Th17 cells may also directly promote autoantibody production by disrupting germinal centers. IL-17 production was shown to desensitize CXCR4 and CXCR5 signaling
causing increased retention of cells in germinal centers. Antibody blockade of IL-17 was shown to alleviate this excessive humoral response [115].

3 Ontogeny, function, and role of innate B1 cells in SLE

Apart from conventional B and T cell responses, a number of other lymphocyte populations are known to play a role in SLE pathogenesis. Of note, a population of innate B1 cells has been characterized to be expanded in a number of SLE mouse models and may contribute to either SLE pathogenesis or suppression.

Innate-like B1 cells comprise a unique population of mostly fetal-derived cells that have been associated with self-reactivity, autoimmunity, and leukemia [116–118]. As a long-lived and self-renewing population, the majority of B1 cells reside in pleural and peritoneal cavities, although a small proportion of cells are known to reside in the spleen. It has been shown that B1 cells traffic from the spleen to peritoneal cavity in a CXCL13 dependent manner [119]. In fact, splenectomy of mice depletes B1 cells in the peritoneal cavity suggesting that renewal of B1 cells relies on the presence of resident precursors in the spleen [120]. B1 cells can be characterized by a unique pattern of expression of a number of cell surface markers and are B220^lo, IgM^hi, IgD^lo, CD43^+, and CD23^lo. B1a and B1b cells can further be subdivided by expression of CD5 [121]. Interestingly, CD5^+ B1a cells were initially discovered in the NZB mouse strain and associated with an increase in autoantibody production [122]. However, B1 cells have been characterized to generally produce low-affinity antibodies and their role in autoimmunity is not entirely clear. These findings are further complicated by the discovery that BCR signaling, similar
to TCR signals, upregulates CD5 expression in an effort to dampen downstream activation cascades. CD5 has been found to be associated with SHP-1/2 expression and is considered as a negative regulator of activation [123]. CD5/- B1 cells, unlike their wildtype counterparts, are capable of generating a strong calcium flux, activation of NFκB, and proliferating in response to BCR cross-linking [124]. As SLE is primarily a B cell-mediated disorder it is important to understand the role that B1 cells, which comprise a unique population of B cells.

3.1 B1 cell development

Unlike adult-derived FO and MZ B2 cells, B1 cell development occurs primarily during fetal development. Although studies have shown that B1 cells can arise later in life from the bone marrow, likely from specialized precursors, the majority of the adult B1 cell pool is fetal-derived and survives through self-renewal. Definitive support for this was provided by experiments with bone marrow versus fetal liver transfers into SCID immune-deficient animals. Fetal liver transfers generated a large proportion of CD5+ B1 cells as opposed to bone marrow or proB cells transfers [125,126]. Recent work from the Dorshkind lab has identified two distinct fetal waves and one adult wave of B1 development [127]. This work has shown a unique transcriptional network controlling the development of B1 and B2 cells, highlighting that these are distinct lineages.

The distinct lineage of B1 cells is further supported by their differentiated heavy chain repertoire, functional responses, and transcriptional profiles. B1 cells frequently express $\text{V}_{	ext{H}11}$ which pairs with $\text{V}_{\kappa9}$ to recognize phosphotidyl choline on aged red blood
cells. This pairing is exceedingly common (5 - 15%) amongst B1 cells but rare in B2 populations [128]. Interestingly, transgenic animals expressing $V_{H}11$ had difficulty generating mature B cells, with a high proportion of splenic B cells expressing endogenous receptors. Further study revealed that the preBCR complex was inefficiently paired with this heavy chain, resulting in attenuated signaling. Unlike B2 cells, B1 cells select on less efficient preBCR complexes explaining the differential usage of heavy and light chains [129,130]. On top of this, fetal B1 cells express less Tdt, the enzyme responsible for germline addition of nucleotides at Ig heavy chain junctions, further constraining the IgH repertoire [121].

Studies with Thy1 and BCR transgenic animals have also suggested that development of B1 cells is reliant on positive selection through the BCR [131]. Additional evidence for this positive selection comes from studies with regulators of BCR signaling: deletion of positive regulators such as CD19 or vav-1 decrease B1 cell number while deletion of negative regulators such as CD22 or SHP-1 increased B1 numbers. This positive selection may explain how the repertoire of B1 cells is maintained to lipids and autoantigens [123].

It has recently been suggested that B cell lymphopoiesis may undergo a developmental switch through the interactions of Lin28b, Let7, and mIR-125b [132,133]. This recent work showed that these regulators control expression of Arid3a, a key mediator of B1a cell development. Arid3a functions by binding $V_{H}$ and impairing BCR
signaling thereby enhancing the output of B1 cells. This regulation may in fact determine fetal B cell development in mice, acting as the critical switch that occurs after birth. Importantly, intact BCR signaling was once again shown to be critical in the development of B1a cells [133].

3.2 B1 cell functions

With all these differences in development, B1 cells are functionally distinct. B1 cells are long-lived, hyporesponsive to stimulation through the BCR, and proliferate in response to phorbol esters. Transcriptional studies revealed that B1 cells constitutively upregulate BLIMP-1 and XBP-1. The limited repertoire of B1 cells allows for the generation of protective autoantibodies early in development. The protective effect of these antibodies is thought to be related to their low affinity polyreactivity which allows for their binding to multiple pathogens, leading to complement-dependent clearance of bacteria [121]. B1 cells can also produce large amounts of IL-10 and are thought to be the main producers of B cell derived IL-10 [134]. It is also interesting to note that in response to stimuli, either BCR dependent or independent, B1 cells migrate to the spleen or mucosal tissues [135,136].

3.3 B1 cells and autoimmunity

By their nature, B1 cell are generally autoreactive and prone to secretion of low affinity IgM autoantibodies. Although, the exact role of B1a cells in autoimmunity has not been resolved. Studies have suggested that B1a cells, through IL-10 production, can actually protect against disease. In the NOD mouse model of diabetes, for instance, B1a cells
were shown to prevent autoimmunity [137]. Neonatal CD5⁺ B cells were also shown to control DC responses following stimulation with TLR9 [121]. On the other hand, B1a cells are expanded in many murine models of SLE suggesting a potential link to disease [118]. Although B1 cells typically produce low-affinity natural IgM autoantibodies, the inflammatory environment of autoimmunity can alter their function. For instance, in NZB/W F1 mice, B1a cells have been found in the kidneys where they produce anti-dsDNA IgG causing damage [138]. Additionally, ablation of the peritoneal B1 compartment by hypotonic treatment reduced the severity of disease, supporting the involvement of these cells in autoimmunity [139]. Peritoneal B1a cells have also been shown to polarize CD4 T cells to Th17 cells and prevent the generation of regulatory T cells [140]. Paradoxically, a study which administered IL-5 to boost B1 cell numbers in NZB/W F1 mice actually reported a decrease in antibody production and nephritis, exemplifying the duality of these cells [141].

3.4 B1 cells in humans

While B1 cells in mice have been extensively characterized, the phenotypic identification of equivalent cells in humans is still under dispute. Numerous groups have identified various markers which may be used to discriminate human B1 cells, with no consensus being reached. A number of studies have shown that CD5⁺ IgM producing B cells can be found in adults [121], however, unlike mice, CD5 does not appear to be a sufficient marker for identifying B1 cells in humans and there is an absence of these cells in the peritoneal cavity [142]. CD5⁺ B cells are found within cord blood and together with those seen in adults, have been reported to correspond to pre-naïve immature B cells as
opposed to a distinct B cell lineage [143]. Nevertheless, rheumatoid arthritis patients were found to have increased CD5⁺ B cell frequencies which when sorted could produce rheumatoid factors [144]. Fc receptor like 4(FCRL4)⁺CD21lo B cells have also been proposed to be human B1 cells based on their functional responses and are expanded in the peripheral blood of SLE patients [121,145]. Similarly, CD27⁺CD43⁺CD70⁺ B cells, also thought to represent human B1 cells based upon their ability to secrete IgM and high CD86 expression, are elevated in the peripheral blood of SLE patients compared to controls [146]. In a recent study, a BLK polymorphism associated with decreased BLK expression was shown to be associated with abnormal B1 cell accumulation in healthy controls [147]. These data suggest that B1-like cells are present in humans and may contribute to the development of autoimmunity possibly through genetic mechanisms that are supported by ongoing inflammation.

4 Role of regulatory T and B cells populations in the suppression of SLE

As alluded to in previous sections, regulatory lymphocyte subsets may help to control SLE pathogenesis. The role of regulatory T cells in suppression of autoimmune diseases, including SLE, has been relatively well characterized, and will be only briefly discussed below. However, within the last decade, an expanding role of regulatory B cells has also been recognized and has become a potential target of therapy. Regulatory B cells have now been identified in numerous B cell compartments and in a number of diseases including cancer and autoimmunity.
4.1 T regulatory cells in SLE

There is considerable evidence that, regulatory T cells can control and prevent autoimmunity. T regulatory cells or Tregs can control B cell responses either through direct interactions or the release of immunosuppressive cytokines such as TGFβ and IL-10. Secretion of these cytokines or other immunomodulating cytokines such as IL-35, granzyme, or perforin, by Tregs has also been shown to control the immune response by altering DC priming and T cell activation, or by directly inducing cell lysis. In addition, Tregs can directly interact with immune cells modulating their function through the action of inhibitory molecules such as CTLA-4 and GITR [148,149]. Tregs can either develop from central thymic precursors or be induced in the periphery. Regardless of their origin, they are generally characterized by expression of the transcription factor Foxp3 and IL-2 receptor alpha chain (CD25). Development of these cells is dependent on the expression of IL-2 and TGFβ. Highlighting the importance of these cells, mouse models with Foxp3 knockouts or mutations result in autoimmune disease [150,151]. Similar studies in humans have identified patients with mutations in Foxp3 that present with severe immune dysregulation [152,153].

The role of Tregs in murine models of SLE has been extensively studied. Studies with NZB/W F1 animals revealed a critical role for Tregs with depletion studies. The depletion of these cells, using an anti-CD25 antibody, resulted in early onset of nephritis and the production of pro-inflammatory cytokines [154]. Additionally, the transfer of in-vitro expanded Treg into NZB/W F1 recipients reduced the incidence of renal disease and
sloved progression [155]. The frequency of these cells is often reduced in lupus-prone animals. In Sle1 mice, for instance, the frequency of Foxp3+ Tregs is reduced and correlated to autoantibody production [156]. In SLE patients, the frequency of regulatory T cells is harder to assess as Foxp3 and CD25 expression is induced in activated T cells. Nevertheless, the majority of studies have identified a loss of CD25hiCD4+ Tregs in patients that inversely correlates with disease activity [148].

4.2 Regulatory B cells
Antigen presentation and antibody production are key features of B cells and have been extensively studied. However, in recent years a possible suppressor role for B cells has been unearthed. One of the first observations suggesting that B cells could suppress autoimmunity came from the study of B cell deficient animals in a mouse model of MS, experimental autoimmune encephalomyelitis. In these µMT deficient animals, recovery of autoimmunity was paradoxically reduced in the absence of B cells, otherwise thought to promote disease [157]. Follow-up work on the idea of suppressor B cells identified IL-10 producing B cells are regulators of colitis and arthritis [158–160]. Since then, numerous groups have worked to characterize the phenotype and function of this B cell population. Although no consensus has been reached and no master regulator of development discovered, this population of B cells is undoubtedly important to the development and control of a number of autoimmune disorders. For the sake of consistency, all suppressor B cell populations, regardless of the phenotypic markers used to identify these cells, will be named regulatory B cells or Bregs in this thesis.
4.2.1 Phenotype and function of regulatory B cells

Unfortunately, many overlapping phenotypes of regulatory B cells have been described in the literature with largely similar function. In mice, regulatory cytokine producing B cells have been documented as T2-MZP (CD19⁺CD21hi⁺CD23hi⁺CD24hi⁺), B10 (CD5⁺CD1dhi⁺), MZ B cells (CD19⁺CD21hi⁺CD23⁻), plasmablasts (CD138⁺CD44hi⁺), and Tim-1⁺ B cells. Furthermore, Treg expanding B cells (GITR⁺) and killer B cells (FasL⁺) have also been shown to have regulatory function in disease. In humans, two populations of regulatory B cells have been characterized as either immature B cells (CD19⁺CD24hi⁺CD38hi⁺) or Br1 (CD19⁺CD25hi⁺CD71hi⁺) [161,162]. The wide array of markers used to discern regulatory B cells and the reliance on phenotypic markers which may be altered due to activation has made it difficult to identify a shared developmental pathway. To date, no one transcription factor, similar to Foxp3 for Tregs [163], has been identified for regulatory B cells. Nevertheless, development of these cells has been postulated to arise in one of two ways. Either regulatory B cells represent a unique population of cells with a unique developmental pathway, similar to Tregs, or B cells are pushed towards a regulatory phenotype given the right environment. The later hypothesis would suggest that a number of B cell subsets could therefore become regulatory. Since gene profiling experiments have failed to identify a shared transcription factor, the idea of induced B regulatory cells is much more likely [164,165].

Given that regulatory B cells can arise from immature and mature B cells as well as plasmablasts, the environment and signals which drive a regulatory phenotype are
critical. It has been shown that TLR and/or CD40 signals can drive the development of regulatory B cells [166]. Recently, a number of publications have also highlighted the role of the inflammatory environments in supporting regulatory B cells. During the course of disease, regulatory B cell numbers increase in response to inflammation and are critical to relieving disease. A recent publication has shown that IL-1β and IL-6 can drive regulatory B cell expansion in a model of arthritis [167]. Absence of IL-1R1 or IL-6R on B cells resulted in reduced B regulatory cell numbers and uncontrolled disease with increased proportions of Th17 cells [168]. Interestingly, both IL-21 and GMCSF, important in TH17 development, have been implicated to be important to regulatory B cell frequencies [169].

Studies have also shown the importance of BCR diversity in the development of regulatory B cells. In MD4 mice, where the BCR is fixed to reactivity with Hen-Egg Lysozyme (HEL) as a result of an anti-HEL Ig transgene, there is a reduction in regulatory B cell frequency and function in response to TLR stimulation [158,170]. Other transgenic B cell models have also been shown to lead to altered frequencies of regulatory B cells. Whether this has to do with impaired development or activation is still unclear. Similarly, deletion of STIM-1 and STIM-2 on B cells, critical for calcium flux, reduced the production of IL-10 in response to antigen stimulation [171].

Most studies of regulatory B cells have characterized their suppressive ability by production of IL-10, a pleiotropic immunomodulatory cytokine. Studies of regulatory B
cells have shown that these cells can limit the proportion of pro-inflammatory T cell subsets, including Th1 and Th17, by influencing DCs [172–174]. A number of studies have also shown that regulatory B cells are critical for Treg maintenance and expansion. In studies of mice with a B cell specific IL-10 conditional knockout, Treg frequencies were reduced. Some work has shown that regulatory B cells might directly interact with Tregs to drive differentiation [175,176]. Apart from IL-10, regulatory B cells have also been shown to secrete TGFβ and IL-35, both of which have been previously implicated in immunosuppression [161]. Lastly regulatory B cells, through the production of cytokines may also induce apoptosis in CD4 T cells and promote anergy in CD8 T cells [177,178]. It has even been suggested that in humans, regulatory B cells may control the proportion of NKT cells [179].

Although most studies have characterized the function of regulatory B cells in the spleen, early work also identified this IL-10 producing population in the peritoneal compartment and more recently regulatory B cells have been noted in draining mesenteric lymph nodes in models of colitis [167]. Although their exact development is still unknown, a multitude of studies supports the idea that regulatory B cells are important players in a number of diseases, including SLE.

4.2.2 Regulatory B cells in SLE

Studies in murine models of spontaneous lupus have also highlighted a role for regulatory B cells in control of disease. Even though B cells are the primary drivers of pathogenesis in SLE, in NZB/W F1 mice early treatment with anti-CD20, a B cell depleting antibody,
exacerbates disease [180]. This finding has been linked to an early expansion of regulatory B cells in young NZB/W F1 mice which is abolished with this treatment. Interestingly, treatment later during disease development has a protective effect. In studies of CD19-/- NZB/W F1 mice, which lack regulatory B10 cells as well as marginal zone B and B1 cells, autoantibody production was significantly delayed but nephritis and mortality increased [181]. Importantly, transfer of splenic B10 cells from wildtype NZB/W F1 mice into CD19 knockout recipients prolonged survival, indicating a possible regulatory effect [181]. In lupus-prone MRL/lpr mice, B cell specific deletion of IL-10 had no impact on disease progression [182]. However, transfer of anti-CD40 stimulated T2-MZP cells could inhibit the development of lupus by inducing IL-10 producing Tregs [183].

As mentioned earlier, a number of regulatory B cell populations have been identified in humans. The first studies on IL-10 producing B cells in SLE identified an increased frequency of these cells following stimulation of PBMCs in vitro with PMA and Ionomycin, as compared to controls [184]. Tedder et al also found an increased population of IL-10 producing regulatory B cells in SLE patients which was localized to the CD27+ memory compartment [185]. However, other studies have found a reduction in regulatory B cell frequencies in these patients. Studies have shown that the frequency of transitional B regulatory cells is reduced in SLE patients. Moreover, the function of these cells in response to CD40 was also reduced suggesting both a numeric and functional abnormality in affected individuals [186]. Interestingly, rituximab treatment resulted in increased frequencies of transitional regulatory B cells. A more recent study has
confirmed this finding, showing a loss of IL-10 producing B regulatory cells in SLE patients which correlated with lupus nephritis progression, indicative of a potential protective role [187].

Recent work from Mauri and colleagues has shown the pDC from healthy individuals induce the differentiation of regulatory B cells in an IFNα dependent manner. This expansion then negatively regulates pDC activation through an IL-10 dependent mechanism controlling inflammation. In SLE patients, increased frequencies of hyperactivated pDCs were found to be associated with decreased B regulatory cell differentiation. As before, patients on rituximab treatment had normalized pDC functionality [188].

5 The role of IL-10 in SLE
The characterization of regulatory T and B cells in murine and human models has been dependent on the production of the IL-10, a potent immunosuppressive cytokine. IL-10 production was originally characterized as a novel secreted factor produced by Th2 cells that inhibited TNFα and IFNγ production. Since then, IL-10 has been shown to be produced by a number of leukocytes including monocytes, macrophages, T helper cells, B cells, and natural killer (NK) cells. IL-10 is a 35kDa dimeric protein with high homology (80%) between mouse and humans. Interestingly, IL-10 is encoded on mouse and human chromosome 1 [189].
The secretion of IL-10 is critical to restraining the immune response. Its importance is highlighted by the spontaneous onset of colitis in IL-10 knockout animals, despite normal antibody responses. Similar effects are seen with blocking antibody studies [190]. IL-10 knockout animals raised in germ-free conditions, however, did not develop colitis, suggesting that activation of an immune response by gut bacteria is critical to disease and that IL-10 is a major player in the control of inflammation [191]. Interestingly, the spontaneous colitis in this model is driven by Th17 and Th1 cells, which become activated as a result of IL-23 production by DCs and macrophages, a process which is normally inhibited by IL-10.

As mentioned earlier, IL-10 can act directly on antigen presenting cells, T cells, and B cells. On macrophages and dendritic cells, IL-10 can suppress the immune response by inhibiting the expression of MHCII molecules and costimulatory proteins like CD80 and CD86 [192]. IL-10 can also inhibit the production of several pro-inflammatory cytokines including IL-1, IL-6, and TNFα as well as IL-10 itself. On T cells, IL-10 can directly inhibit the activation of some populations. Although expression of IL-10R is low on memory and activated T cells, studies have shown that during a viral infection IL-10 production inhibits the development of memory CD4 T cells [193]. IL-10 is also a potent growth factor for B cells and can promote survival, differentiation, antibody production and MHCII expression. These contradictory roles as both a critical B cell factor and immune suppressor have made the establishment of the precise role of IL-10 in SLE difficult [194].
5.1 IL-10 in SLE

In murine models of SLE, the role of IL-10 has been conflicting with both pathogenic and protective roles being demonstrated. In NZB/W F1 mice, the continuous administration of anti-IL-10 antibody delays the development of disease [195]. However, in NZM-derived \( \text{Sle1, Sle2, and Sle3} \) triple congenic animals, continuous low level expression of IL-10 delayed the production of autoantibodies and kidney damage [196]. In contrast, in SLE patients, serum IL-10 levels are consistently reported as elevated and have been correlated with disease activity [197–199]. It has been hypothesized that these elevated levels of IL-10 may contribute to disease by promoting B cell activation and antibody production. In support of this hypothesis, \textit{in vitro} IL-10 depletion studies with anti-IL-10 decreased autoantibody production. Furthermore, a pilot clinical trial with anti-IL-10 depletion therapy resulted in clinical improvement in SLE patients [200]. To reconcile these differences, as IL-10 is known to be an immunosuppressive cytokine, studies have found that IL-10 from SLE patients may be less effective at reducing TNF\( \alpha \) and IL-6 levels [201].

6 Invariant Natural Killer T cells as mediators of the immune response

In the original studies of chromosome 4 NZB congenic animals, a previously undocumented expansion of iNKT cells was discovered [72]. Acting as central orchestrators of the immune response, the role of these cells in autoimmune diseases remains unresolved. Whether pathogenic or suppressive, iNKT cells undergo a unique
developmental pathway that allows these cells to respond rapidly to direct and indirect stimulation.

Natural Killer T cells, originally named for their expression of NK cell markers and cytotoxic activity, are innate-like lymphocytes that recognizes glycolipid presented on CD1d, an MHC like molecule [202]. Type I NKT cells, identified by staining with αGalCer-loaded CD1d tetramers, express a semi-invariant TCR with Vα14Jα18 alpha chain paired with a limited repertoire of beta chains, usually Vβ8.2, Vβ7, or Vβ2. This unique pairing allows for NKT cells to rapidly recognize glycolipids and produce a range of immunomodulatory cytokines immediately after stimulation. NKT cells have been implicated in a number of diseases including cancer, autoimmunity, infection, allergy, and GVHD through the production of a number of critical cytokines including IFNγ, IL-4, TNFα, IL-17, and IL-10, to name a few. Type II NKT cells are NKT cells whose TCR does not bind αGalCer-loaded CD1d tetramers. These cells recognize a number of lipid antigens including sulfatide and lysophosphatidylcholine also presented on CD1d. Similar to type I NKT cells, their TCR repertoire is restricted. Importantly, CD1d knockout animals, remove both type I and II NKT cells [203–205].

In humans, the repertoire of NKT cells is complicated by the presence of three additional CD1 lipid presenting molecules including CD1a, CD1b, and CD1c. CD1e is another lipid binding molecule, which is not found on the cell surface but is instead involved in lipid transport within the cell. Human type I NKT cells that develop on CD1d
also express a semi-invariant TCR with Vα24Jα18 and Vβ11 chains and can be identified with αGalCer-loaded human CD1d tetramers. In contrast, T cells that develop on CD1a-c are highly diverse and include both αβ and γδ T cells [203–205].

6.1 Invariant NKT cell development and function

Like conventional T cells, NKT cells develop in the thymus and are dependent on TCR rearrangement. Mice lacking RAG-1 or RAG-2 fail to produce NKT cells [206]. However, unlike T cells which develop on antigen presenting thymic epithelial cells, NKT cells begin to differentiate at the double positive stage of development in response to homotypic interactions. In fact, deletion of genes which extend the lifespan of DP thymocytes, including BCL-xL and RORγT, decrease the frequency of NKT cells [207,208]. As developing T cells recombine to randomly form an invariant TCR, recognition of self-lipid presented on CD1d from other double positive thymocytes drives differentiation to the NKT cell lineage. The exact identity of this self-lipid has yet to be determined but some studies have implicated isoglobotrihexosylceramide (iGb3) [209]. However, in studies of iGb3 synthase knockout animals, required for the production of iGb3, NKT cell development was unaffected [210].

Four distinct stages of NKT cell development, stage 0-4, have been identified based on the expression of the cell-surface markers CD24, CD44, and NK1.1. Stage 0, characterized by CD24\(^+\)CD44\(^{lo}\)NK1.1\(^{lo}\) cells represents the most immature stage of development. As developing cells proliferate, they lose CD24 expression and enter stage 1. With continued proliferation, expression of CD44 the typical memory/activated T cell
maker, is acquired in the thymus (stage 2). As proliferation slows, NKT cells finish their development/maturation and acquire expression of NK1.1. It is interesting to note that most NKT cells leave the thymus at stage 2 of maturation, before acquiring expression of NK1.1 [211].

Recent work indicates that the different stages of NKT cell development and maturation can be discriminated by expression of transcription factors, PLZF, GATA3, RORγT, T-bet, and Erg2 (Figure 2). In addition, studies have been able to delineate different functional subsets of NKT cells, NKT1, NKT2, and NKT17, based on the expression of PLZF, GATA3, RORγT, and T-bet, which have been shown to have distinct patterns of tissue distribution and function [211–213]. NKT2 cells, for instance, are found primarily in lung tissues and are thought to be associated with asthma and acute hypersensitivity. RNA sequencing efforts have confirmed that NKT1, NKT2, and NKT17 are indeed distinct in their gene expression profiles and unsurprisingly share overlap with growing field of innate lymphoid cells (ILC) subsets, ILC1, ILC2, and ILC3, respectively [214,215]. Interestingly, the proportion of these NKT subsets and overall NKT cell frequencies are highly variable between inbred mouse strains [212].

One key signaling cascade responsible for NKT cell lineage commitment is transmitted through the SLAM family of molecules. SLAM molecules, discussed in greater detail in following sections, can transduce both positive and negative signals
Figure 2: iNKT cell development diverges into three distinct subsets as identified by cell-surface markers and transcriptional factors

Representative scheme outlining the development of iNKT cells from DP thymocytes to NKT1, NKT2, and NKT17. Markers used to delineate iNKT cell subsets are defined. Reproduced with permission from [216].
using a number of adaptor proteins. In studies of SAP, a downstream adaptor of SLAM proteins, knockout animals were discovered to lack NKT cells[217]. This condition is mimicked in SAP-deficient patients who present with a rare form of autoimmunity and also lack peripheral NKT cells [218]. A number of studies have shown that several SLAM family molecules and SLAM signaling are critical to driving NKT cell development. These include Slamf1(CD150), Slamf6(Ly108), and Slamf3(Ly9) [219,220]. SLAM family signaling is required for induction of Erg2 and PLZF, two critical transcription factors involved in NKT cell development and maturation [221–223]. Erg2 deficient animals have a clear blockage in NKT cell development with an absence of mature stage 1-3 cells [221]. It has been suggested that PLZF, though expressed in a number of innate-like cell populations, is the most critical transcription factor required for NKT cell development. In PLZF deficient animals, NKT cell numbers are significantly reduced and the remaining cells fail to acquire their typical memory/activated phenotype suggestive of a serious blockade in development [222].

There is evidence that NKT cells are activated by self or microbial lipids. Bacteria such as *Sphingomonas*, *Ehrlichia* and *Borrelia burgdorferi* express α-linked glycosphingolipids and diacylglycerol products that can be directly recognized by iNKT cells [204]. Self-lipids, such as iGB3, may also be presented, modified, or increased in production during an immune response acting as cognate antigens for activation [204]. Apart from this TCR/CD1d dependent activation, NKT cells may also be activated indirectly by cytokine production. In studies with *Escherichia coli* LPS, activation of
NKT cells did not require CD1d but instead was reliant on IL-12 and IL-18 production by dendritic cells [224]. Broadly speaking, cytokines can modulate the NKT cell response and either drive subsets of NKT cell activation or enhance stimulation. Additionally, co-stimulation through CD28 or 41BB is required for effective IL-4 and IFNγ production. CD40, OX40, and CXCR6 ligation has also been implicated in specifically promoting IFNγ production in NKT cells [204]. Thus, both the inflammatory milieu and antigen recognized appear to influence NKT cell function.

6.2 Invariant NKT cells in autoimmunity
Defects in NKT cell frequency and function have been implicated in a number of mouse models of cancer and autoimmunity. In fact, in BALB/c mice with a Jα18 knockout that lack type 1 NKT cells, aged mice develop a lupus-like disorder characterized by production of anti-dsDNA antibodies, complement activation, and nephritis [225]. While this observation supports a role for NKT cells in the regulation of autoimmunity, results in the various autoimmune mouse strains and models have been contradictory, making it difficult to establish the exact role of these cells in the development of autoimmunity. In pristane-induced models of SLE, the effects of NKT cell activation can vary depending on the genetic background. In BALB/c mice, treatment with αGalCer protects mice against nephritis by inducing a Th2 response. However, in SJL/J mice, treatment with the same potent ligand promotes a Th1 response and exacerbates disease. The differences in outcome in these mice appear to be related to the cytokines produced, which in turn are dependent on the genetic background of the animals [226].
In the prototypic NZB/W F1 SLE model, NKT cells have been found in the inflamed organs and shown to induce autoantibody production. The NKT cells in these mice were characterized as hyperresponsive, producing greater amounts of inflammatory cytokines [227,228]. Along these lines, treatment with blocking anti-CD1d antibody decreased disease severity and repeated injection of αGalCer worsened disease [229]. Yet paradoxically, knockout of CD1d exacerbated disease in the same animals [230], suggestive of a protective effect. Studies in NZB/W F1 mice have also suggested that Ly108 hi NKT cells, which are expanded, are prone to IL-21 and IL-17 production and may represent a more pathogenic NKT subset related to autoimmunity. Similarly, transfer of NKT cells from aged mice into young NZB/W F1 recipients accelerated disease by increasing producing to anti-dsDNA antibodies. In vitro studies from these animals suggest that NKT cells can directly stimulate autoantibody production in B cells [231].

Recently, direct evidence for an interaction between CD1d expression on B cells and NKT cells has been uncovered in an apoptotic debris injection model of SLE. In this study, reduction of NKT cells led to increased activation of autoreactive B cells. The presence of NKT cells was able to prevent germinal center entry of B cells, acting at a crucial immunoregulatory checkpoint [234]. However, as this model is dependent on the
injection of high doses of apoptotic cells that alter the NKT cell phenotype, these results may not apply to spontaneous SLE models.

6.3 Invariant NKT cells in humans

The study of NKT cells in human disease has been limited by the low frequency and high variability of these cells in peripheral blood. The average frequency of NKT cells is approximately 0.01% of total peripheral blood mononuclear cells and can vary 100 fold between individuals. Still, in SLE patients, the number and function of NKT cells was discovered to be lower and correlated with disease activity [235]. More telling is that NKT cell numbers were restored when patients were treated with corticosteroids or rituximab [236,237]. Functionally, it has been noted that proliferation of NKT cells in response to αGalCer is defective in SLE patients as compared to healthy controls and some studies identified increased apoptosis as a factor [235]. Still, the question remains whether these defects in NKT cell function and frequency are a consequence or a driver of disease. As a hint, one study found that in first-degree relatives of SLE patients, NKT cell numbers were decreased and correlated with ANA positivity suggesting that this phenotype may precede clinical autoimmunity [238].

7 The SLAM family of receptors and their role in SLE pathogenesis

As mentioned, SLAM receptor signaling is critical to iNKT cell development in the thymus. However, the role of these receptors is broad and not limited to this subset.
Indeed, the SLAM receptors and their downstream adaptors have diverse effects on T and B cell function which have been implicated in the development of autoimmunity.

The signaling lymphocytic activation molecule (SLAM) family of proteins comprises a group of type 1 transmembrane proteins expressed exclusively on a broad range of immune cells. The family has 9 members (SLAMF1-9) encoded on mouse and human chromosome 1 in a tightly linked region (Figure 3). All of the proteins are composed of two extracellular Ig-like domains, except for Ly9 which has four domains, and a transmembrane region with intracellular tyrosine associated signaling motifs (Figure 4). With the exception of CD244(2B4, SLAMF4) that binds to CD48(SLAMF2), this family of proteins are self-ligands that are engaged through homotypic interactions [239].

The SLAM family of molecules likely arose through the duplication of a single gene. Polymorphisms in the locus and within individual genes have been shown to result in differential splice variant expression and are associated with disease. Differences in Ly108(Slamf6) that alter the expression of differential splice variants have been linked to murine SLE [240,241]. Similarly, expression variations in Ly9(Slamf3) and 2B4(Slamf4) have been linked to SLE and RA in humans [242,243]. By modulating signaling cascades, the SLAM family of proteins has recently come into focus as a critical modulator of the immune response. The important role of SLAM molecules is highlighted by cases of human X-linked lymphoproliferative disease (XLP) characterized
Figure 3: Genomic organization of SLAM receptors

Representation of the genetic loci encompassing the SLAM family of receptors in mice and humans. Reproduced with permission from [239].
Figure 4: Mouse and human SLAM receptors
Representative summary of the SLAM family of receptors and corresponding ITSM identified in mice and humans. Reproduced with permission from [239].
by an ineffective response to EBV. In 60 to 70% of cases, this rare disease is associated with deleterious mutations in SAP, a key downstream signaling molecule of SLAM family proteins. Patients typically present with accumulation of immune cells and an increased risk of malignant lymphomas. Studies have revealed that functions of B, T, and NK, and NKT cells are all defective in these patients [218]. Furthermore, the Sle1b locus which is associated with lupus encompasses the entire SLAM family and studies have identified Ly108 as a key regulator of autoimmunity [240]. Similar studies of the Nkt1 locus derived from NOD autoimmune diabetes-prone mice found a correlation between disease development and the SLAM family of proteins, highlighting the importance of these molecules in a number of diseases [244].

The first studies of SLAM receptors and their roles in immune responses came from studies of 2B4, Ly108(or NTB-A in humans), and CRACC(Slamf7). Crosslinking antibodies to these receptors were discovered to stimulate IFNγ production and spur killing by NK and T cells [245–247]. Similar studies with T cells discovered that crosslinking CD150(SLAM, Slamf1) could promote secretion of IFNγ following CD3 stimulation [248]. B cell proliferation in response to IL-4 and CD40 was also shown to be modulated by CD150 engagement [249]. Of course, these effects were not limited to T and B cells and were later to the same molecules were shown to modulate critical signaling events in macrophages and DCs [250]. Clearly, SLAM receptor signaling can have a diverse range of effects on a number of immune populations.
7.1 Activation of SLAM family receptors

Like a number of other immune signaling molecules, the SLAM family of receptors relies on the binding of adaptor proteins to immunoreceptor tyrosine-based switch motifs (ITSMs) located in their cytoplasmic domains [251]. The binding of SAP, EAT-2 and ERT (in mice) or SHP-1, SHP-2, or SHIP-1 modulate downstream signals by enhancing or dampening phosphorylation events, respectively.

As mentioned earlier, the first noted function of SLAM receptors was their ability to stimulate cytokine production in conjugation with TCR stimulation in T and NK cells. However, as these studies relied on antibody-mediated assays, their exact role was difficult to discern. For example, antibodies against CD150 were shown to enhance T cell stimulation yet studies with artificial APCs expressing CD150 reduced cytokine production [252,253]. This finding suggests that some antibodies may be acting to block interaction as opposed to inducing signaling. This is supported by studies from SAP knockout animals which reported an increase in IFNγ production upon stimulation [254]. However, these same studies identified a definite loss of IL-4 production and Th2 function, with normal proliferation and IL-2 production. When SAP deficient mice were crossed with IFNγ knockout mice, the loss of IL-4 production was retained, suggesting a role for SLAM molecules in Th2 differentiation [253]. This Th2 defect was confirmed in SAP knockout animals infected with Leishmania major. In this model, Th2 responses are critical to supporting infection and so SAP deficient animals were shown to be resistant to colonization [254]. Consistent with these findings, CD150 knockout animals had
reduced IL-4 production and were resistant to Th2-mediated allergic inflammation [255,256]. Ly108 knockout animals revealed a loss of IL-4 production as well as a defect in neutrophil function and NKT cell development [257]. Similarly, Ly9 knockout animals had a loss of IL-4 [220]. In summary, although the effect on IFNγ production remains contentious, the SLAM receptors have a clear role in eliciting IL-4 mediated immunity.

The absence of SAP has been linked to severe defects in the humoral response. In XLP patients there is a pronounced loss of antibody-mediated responses and SAP knockout animals exhibit a similar defect in germinal center reactions [258]. Interestingly, immunization of SAP knockout mice with T-independent antigens results in normal responses. However, immunization with T dependent antigens results in defective antibody generation with impaired B cell proliferation and germinal center formation [259]. Transfer of wildtype CD4 T cells, and not SAP deficient T cells, could restore antibody production, suggesting that this defect predominantly affected the T cell compartment. It was noted that SAP deficiency leads to a decrease in expression of costimulatory molecules, ICOS and CD40L, required for proper B and T cell interaction. Similar defective responses in the absence of SAP were noted in pathogen infection models [239]. Overall, SAP deficient animals have reduced numbers of memory B cells and plasma cells indicating a clear disruption of the germinal center response [239]. Although studies have shown a clear defect in germinal center responses in SAP deficient animals, the exact role of SAP signaling in B cells is unclear. SAP expression in B cells has been identified but is limited and may be linked to activation. It has been suggested,
instead, that SAP is required for promoting cognate T and B cell interactions. Following immunization, it has been shown that SAP does not play a role in CD4 T cell interaction with DCs, priming, or activation [258]. However, SAP expression was absolutely critical to T-B cell conjugate formation. In the absence of this adapter, T and B cells could not form long-lasting interactions [258]. As a consequence, since B cell contact is required for Tfh cell formation, it has been shown that SAP deficient CD4 T cells fail to differentiate towards this effector lineage.

Although the adaptor, SAP, has been extensively shown to play a critical role in the immune response, the individual contribution of the SLAM receptors is still not well-defined. The expression of several SLAM receptors has been identified on T and B cells. CD84, Ly108, and Ly9 have been shown to be upregulated on Tfh cells. Similarly, CD84 and Ly108 are also known to be upregulated on GC B cells [260]. Initial studies with CD84 deficient mice identified that this molecule was critical to germinal center reactions [260,261]. These studies also revealed that Ly108 expression was critical to T-B cell interactions, suggesting that these two molecules play a critical role in germinal center formation. However, a recent publication with triple knockout (CD150, Ly108, and CD84) animals failed to recapitulate a loss of germinal centers or a defect in IL-4 production [262]. Differences from these studies may be due to incomplete backcrosses in the initial studies, as knockouts prior to CRIPSR technologies were created on the 129 background. As discussed below, there are large variations in the SLAM family of
receptors between inbred strains of mice and incomplete backcrossing may have resulted in the retention of some unwanted polymorphisms.

7.2 Splice isoforms of SLAM Receptors and genetic haplotypes
Several isoforms of SLAM receptors, including CD150, Ly108, CD84, Ly9, and CRACC, have been discovered [239]. These isoforms have been shown to differ in the length of their cytoplasmic domain which can result in additional immunoreceptor tyrosine-based switch motifs (ITSMs) [239]. Ly108, for example, has three splicing isoforms, Ly108-1, Ly108-2, and Ly108-H1, which all have differential signaling capacity [240,263]. Interestingly, differential expression of these variants has been linked to autoimmunity [241]. In addition, different haplotypes have been identified which segregate with differences in SLAM receptor expression between inbred strains of mice. Ly9, for instance has two alleles. Ly9.1 which is expressed in most inbred strains and Ly9.2 which is expressed in B6 and related animals [264]. On top of this, sequencing efforts have identified numerous polymorphisms within inbred strains of mice, suggesting that these regions are highly variable [265].

7.3 SLAM receptors in autoimmune diseases.
The strongest evidence supporting the role of SLAM receptors in autoimmunity comes from studies of Sle1b mice. These lupus-prone animals contain the shortest interval known to contain the SLAM family of receptors. Studies have shown that the total expression as well as specific expression of splice isoforms is greatly altered in these animals and contributes to significant autoimmunity. In fact, this interval alone is
sufficient to cause high titre ANA production [59,266,267]. In seminal work from 2006, this NZW-derived interval was shown to upregulate Ly108.1 expression which could promote autoimmunity by impairing B cell anergy, receptor editing and deletion [268]. In follow up work, Ly108-1 was shown to better recruit SAP and Fyn to induce stronger downstream signaling cascades, providing a possible explanation for T cell defects [269]. This same interval has also been shown to exacerbates autoimmunity in Fas/lpr animals which results in hyperactivation of the PI3K/AKT/mTOR pathway [270].

However, in similar studies in a cGVHD model of SLE, total loss of Ly108 on the inducing CD4 T cells resulted in exacerbated autoimmune disease. Alternatively, the authors of this study showed that administration of an anti-Ly108 antibody could also ameliorate disease [271]. Interestingly, in this same model of cGVHD, the dual knockout of CD150 and Ly108 reversed this increased autoimmune phenotype [271]. This finding suggests that Ly108 is inherently a negative signal on autoreactive T cells but is complicated by the presence of three distinct Ly108 isoforms, each with differential signal capacity. In fact, Ly108H-1 has been shown to be protective in disease [240].

In humans, Ly9 is the only SLAM receptor to be identified in genome wide association studies of SLE. Nevertheless, two recent publications have found higher expression of NTB-A (Ly108) in SLE patient T cells with an associated increase in signaling and Th17 frequencies [272,273]. These studies showed that SLAMF6 signaling on human T cells could promote Th17 differentiation suggesting a possible causative role
in SLE pathogenesis. Together, these finding from congeneric mouse models, knockouts, and patients provide clear evidence for the role of SLAM receptors in autoimmunity.
THESIS OBJECTIVES

SLE is a complex and multifactorial autoimmune disease with a strong genetic basis. As outlined in Chapter I, the pathogenesis of this disease relies on the complex interaction of numerous immune cell types which act in concert to support the production of tissue-depositing autoantibodies. This process relies on the breakdown of normal tolerance and control checkpoints which aim to restrain autoimmunity. By using congenic inbred mice, studies can unravel the genetics of autoimmunity which contribute to these breaches. Mapping studies have shown that genes on NZB and NZW chromosome 1 and 4 can promote disease through a number of mechanisms. Interestingly, it was discovered that introgression of NZB chromosome 4 onto an autoimmune background resulted in disease suppression. It was noted that these suppressed mice had expansions of two innate lymphoid populations, CD5+ B and iNKT cells, which have been proposed to regulate autoimmunity. The main objective of this thesis is to use various congenic lupus-prone animals to unravel this suppressed phenotype and characterize the role and function of B1a and iNKT cells.

In Chapter II, the contributions of CD5+ B cells and iNKT cells to autoimmunity were investigated through the use of CD1d knockout animals and adoptive transfer approaches. It was discovered that CD5+ B cells and not iNKT cells were responsible for disease suppression in bicongenic B6.NZBc1c4 animals. Adoptive transfer of CD5+ B cells from B6.NZBc4 animals highlighted their ability to reduce the frequency of pro-inflammatory T cells and possibly control disease. The work raises the possibility that the
expanded B1a compartment may be acting as a regulatory B cell population. Interestingly, this work suggested that genes on chromosome 4 may promote either the expansion or differentiation of regulatory B cells.

To understand the exact mechanisms of action and further localize this suppressive phenotype, additional subcongenic strains and IL-10 knockout mice were generated and phenotyped in Chapter III. This work revealed that expansion of CD5+ B cells was not necessary for suppression in B6.NZBc1c4t mice and highlighted the critical role for IL-10 in maintaining the homeostatic expansion of this population, both in the spleen and peritoneal cavity. Interestingly, a previously unrecognized role for IL-10 in the expansion of iNKT cells was also identified but not in full length B6.NZBc4 mice. Whether this cytokine acts directly to promote survival or proliferation of these populations or through indirect mechanisms is not yet known.

Since knockout of iNKT cells had little effect on disease progression in B6.NZBc1c4 mice, the development and function of these cells was investigated in Chapter IV to explain their relative unimportance. It was discovered that polymorphisms in the SLAM family of receptors cause abnormal iNKT cell development and functional hypo-responsiveness in the periphery. This loss-of-function was specifically shown to result from a loss of trans-Ly108 interactions between DC and iNKT cells. This finding can explain how iNKT cells from B6.NZBc1c4 mice could be expanded in the periphery but have no effect on the autoimmune process owing to their functional abnormality.
Ultimately, understanding how genetic polymorphisms contribute to the expansion and function of CD5+ B and iNKT cells and their relative contribution to SLE pathogenesis is vital to understanding their role in disease, leading to a clearer picture of pathogenesis and improved therapeutics for patients.
CHAPTER II

Suppression of autoimmunity by CD5⁺ IL-10-producing B cells in lupus-prone mice

Yuriy Baglaenko¹,², Kieran Patricia Manion¹,², Nan-Hua Chang¹, Christina Loh¹,²,³, Ginette Lajoie⁴,⁵,⁶, Joan E. Wither¹,²,⁷

¹Arthritis Center of Excellence, Toronto Western Hospital, Toronto, Ontario, Canada; ²Department of Immunology, University of Toronto, Toronto, Ontario, Canada; ³Department of Microbiology and Immunology, Stanford University, Stanford, California, USA(Current Address of CL); ⁴Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada; ⁵Department of Pathology, Mount Sinai Hospital, Toronto, Ontario, Canada; ⁶Department of Pathology, William Osler Health System, Brampton, Ontario, Canada; and ⁷Department of Medicine, University of Toronto, Toronto, Ontario, Canada

(All experiments were performed by Y. Baglaenko; kidney pathology was assessed by G.Lajoie; all other authors assisted with experiments or helped with experimental design and analysis)

Published in Genes and Immunity

July 2015, pp. 311-20.

© Copyright 2015. Nature Publishing Group
Abstract

Systemic lupus erythematosus is a complex autoimmune disorder characterized by the production of pathogenic anti-nuclear antibodies. Previous work from our laboratory has shown that the introgression of a New Zealand Black derived chromosome 4 interval onto a lupus-prone background suppresses disease. Interestingly, the same genetic interval promoted the expansion of both Natural Killer T and CD5⁺ B cells in suppressed mice. In this study, we show that ablation of iNKT cells with a CD1d knockout had no impact on either the suppression of lupus or the expansion of CD5⁺ B cells. On the other hand, suppressed mice had an expanded population of IL-10-producing B cells that predominantly localized to the CD5⁺ CD1dlow compartment. The expansion of CD5⁺ B cells negatively correlated with the frequency of pro-inflammatory IL-17A-producing T cells and kidney damage. Adoptive transfer with a single injection of total B cells with an enriched CD5⁺ compartment reduced the frequency of memory/activated, IFNγ-producing, and IL-17A-producing CD4 T cells but did not significantly reduce autoantibody levels. Taken together, these data suggest that the expansion of CD5⁺ IL-10-producing B cells and not iNKT cells protects against lupus in these mice, by limiting the expansion of pro-inflammatory IL-17A- and IFNγ-producing CD4 T cells.
Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder characterized by the production of pathogenic anti-nuclear autoantibodies (ANA). Previous murine studies indicate that SLE is a complex genetic disease, in which development of disease reflects the balance between susceptibility and suppressor loci. Although studies of congenic mouse strains with introgressed homozygous intervals derived from lupus-prone mouse strains have provided considerable insight into how susceptibility loci promote disease development, less is known regarding the mechanisms involved in inhibition of autoimmunity by suppressor loci. Our laboratory has previously shown that introgression of a New Zealand Black (NZB) homozygous chromosome (c) 4 interval (B6.NZBc4), spanning 32 to 150Mb, onto a C57BL/6 (B6) congenic mouse strain with a homozygous NZB c1 interval (B6.NZBc1) significantly attenuated the fatal autoimmunity observed in this mouse strain [71,274], indicating the presence of a suppressor locus on NZB c4 [74]. Examination of the immune system in B6.NZBc4 mice revealed expansion of two innate-like lymphocyte populations, invariant Natural Killer T (iNKT) cells and CD5⁺ B cells, leading us to hypothesize that one or both of these populations inhibit autoimmunity in B6.NZBc1c4 bicongenic mice. This conjecture was based on studies showing that the number and/or activation of iNKT cells, invariant-like cells that recognize glycolipids presented on CD1d can modulate autoimmune responses. However, the exact role of iNKT cells in autoimmunity remains somewhat contentious, with opposing roles identified in different models of disease. For example, in the (NZB x New Zealand White (NZW))F₁ mouse model of SLE, activation of iNKT cells by injection of αGalCer
exacerbated autoimmunity by induction of a Th1 response that promoted production of pathogenic IgG2a autoantibodies [229]. Supporting a role for these cells in augmenting disease, invariant NKT cells have also been shown to directly interact with autoreactive B cells to promote autoantibody production [227,230]. In contrast, production of autoantibodies was increased in the absence of iNKT cells in a similar model of SLE, suggesting that these cells may sometimes limit rather than enhance the autoimmune response [234]. Studies on SLE patients have also revealed defects in iNKT cell number and function, suggesting a link to disease progression [235].

In addition to iNKT cells, the innate CD5⁺ B cell population has also been shown to play a regulatory role in autoimmunity. This followed the discovery that a subset of these cells, that produces IL-10, can inhibit autoimmune disease. These newly-defined regulatory cells, termed B10 or Breg, have been shown to express a number of cellular markers, but generally localize to the CD5⁺ CD1d.highlight B cell compartment [275]. There has been disparity in the literature regarding both the ontogeny and phenotypic profile of Breg cells [276,277], with numerous studies showing an essential role for IL-10 in the suppressive ability of these cells [278]. Although IL-10 is a pleiotropic cytokine that has numerous effects on different immune populations, suppression of autoimmunity by IL-10 is usually mediated through the inhibition of dendritic cell maturation and antigen presentation or by direct effects on T cell proliferation and differentiation [279]. In recent years, several groups have identified populations of IL-10-producing B cells in various autoimmune mouse models with the capacity to inhibit disease through
suppression of pro-inflammatory T cells. In mouse models of colitis, the transfer of peritoneal IL-10-producing B10 cells was shown to reduce disease severity by suppressing CD4 T cell activation and cytokine secretion[280]. Similarly, in a collagen induced model of arthritis, IL-10 production by B cells was shown to be critical to suppressing Th1 and Th17 responses and attenuating disease [281,282]. These findings contrast with a study in the MRL./lpr mouse model of SLE, where IL-10 deletion in B cells was shown to have no effect on disease [182]. While these results might suggest that regulatory B cells do not play a role in lupus, an a emerging body of research supports the role of IL-10-producing B cells in controlling systemic autoimmunity [180,181,283,284]. This research also extends to SLE patient studies, which have demonstrated that altered Breg function may contribute to lupus pathogenesis in humans [186,285–288].

In this study, we have sought to determine the suppressive capacity of the expanded iNKT and CD5+ B cell populations in our congenic mouse models. Through knockout of iNKT cells, we show that iNKT cells are dispensable for the inhibition of autoimmunity in B6.NZBc1c4 bicongenic mice. In contrast, the expanded CD5+ B cell population in these mice was shown to be highly IL-10 competent and the size of this population correlated with suppression of autoimmunity in NZB.c1c4 mice. Using an adoptive transfer model, we show that these B cells appear to act by inhibition of activation and expansion of pro-inflammatory Th1 and Th17 subsets. Our results suggest Breg cells play a similar role in lupus to other autoimmune diseases and may attenuate disease by limiting T cell help for pathogenic autoantibody production.
Materials and Methods

**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 experiments performed in this study were approved by the Animal Care Committee of the University Health Network (Animal Use Protocol 123).

**Mice.** B6 and NZB mice were purchased from Taconic (Germantown, NY) and Harlan Sprague Dawley (Blackthorn, England), respectively. Congenic mice were generated as previously described [74]. B6.CD1d<sup>−/−</sup> and B6.Thy1<sup>a</sup>IgH<sup>a</sup> mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and bred onto the various congenic backgrounds, using primer assisted breeding. Only female mice were used for experiments in this study.

**Flow Cytometry and Cell Sorting.** Splenocytes were harvested and RBC removed, as previously described [74]. Half a million cells were incubated with mouse IgG (Sigma-Aldrich) for 15 minutes on ice prior to staining with various combinations of directly conjugated mAbs for 30 minutes on ice. The following antibodies were used for primary staining (all purchased from BioLegend or BD Biosciences): FITC-conjugated anti-TCRβ(H57-597), -CD3ε(145-2C11), -CD1d(1B1), -CD62L(MEL-14), -CD23(B3B4), -IgM<sup>a</sup>(MA-69), -CD44(1M7), and -CD4(GK1.5); PE-conjugated anti-NK1.1(PK136), -CD24(M1/69), -CD5(53-7.3), -CD44(1M7), -IgM<sup>b</sup>(AF6-78), and -B220(RA3-6B2); PE-Cy7 anti-CD19(6D5), -CD8(53-6.7), -CD138(MI15), and -CD44(1M7); PerCP-CY5.5 conjugated anti-CD3ε(145-2C11) and -CD4(GK1.5); Allophycocyanin-conjugated anti-CD21(7E9), -CD19(6D5), -CD5(53-7.3), and -CD25(3C7); BV605 conjugated anti-
B220(RA3-6B2) and -CD3 ε(145-2C11); Pacific Blue-conjugated anti-CD4(GK1.5) and -B220(RA3-6B2); Biotin-conjugated anti-NK1.1(PK136) and -CD19(6D5). Allophycocyanin-, PerCP-CY5.5-, Pacific Blue- or Allophycocyanin-Cy7- conjugated streptavidin, purchased from BioLegend, was used for secondary staining of biotinylated mAbs. Dead cells were identified by staining with 0.6µg/mL Propidium Iodide (Sigma Aldrich). Allophycocyanin-conjugated unloaded and PBS-57–loaded mouse CD1d tetramers were generously provided by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). Events were collected on a BD LSRII or BD FACSCanto and analyzed using FlowJo software (Tree Star Inc).

**T regulatory (Treg) Cell Staining.** For Treg staining, RBC-depleted splenocytes were stained for extracellular markers, as described above. After staining, cells were fixed and permeabilized with Foxp3 fixation/permeabilization buffer (Ebiosciences), washed, and stained with PE-conjugated anti-Foxp3 (FJK-16s).

**Detection of cytokine-secreting T cells.** To examine IFNγ and IL-17A production, 0.5 x 10⁶ RBC-depleted splenocytes were stimulated in 96 well flat bottom plates with PMA (50ng/mL, Sigma Aldrich) and Ionomycin (500ng/mL, Sigma Aldrich) in complete media (10% FBS/RPMI1640 plus additives) together with GolgiStop (BD Biosciences) for 4-5 hours at 37°C. After stimulation, cells were transferred to 96 well V-bottom plates and stained with Near Infrared Live/Dead stain (Life Technologies) followed by extracellular staining, as described above. Cells were then fixed with Cytofix/Cytoperm (BD Biosciences). Following fixation, cells were stained for cytokine accumulation with
PE-conjugated anti-IL-17A (TC11-18H10.41) and allophycocyanin-conjugated anti-IFNγ (XMG1.2) for 30 minutes on ice.

**Detection of IL-10-producing B cells.** IL-10 production by B cells was examined under various stimulation conditions. Unless otherwise stated, 0.5 x 10^6 RBC-depleted splenocytes were plated in 96 well flat bottom plates and stimulated for 4-5 hours with PMA (50 ng/mL), Ionomycin (500 ng/mL), and LPS (10 µg/mL, Sigma Aldrich, E-coli 011:B4) in the presence of GolgiStop. Following stimulation, cells were stained with Near Infrared Live/Dead stain and various conjugated antibodies against extracellular markers, and then fixed and permeabilized, as described above. The cells were then stained with allophycocyanin-conjugated anti-IL-10(JES5-16E3) for 30 minutes on ice. In some experiments, the splenocytes were cultured overnight with LPS (10 µg/mL), Imiquimod (2 µM), Poly(I:C) (50 µg/mL), or CpG ODN 1826 (250 nM); all except LPS were purchased from InvivoGen. GolgiStop was added for the last 3 hours of culture and then the cells were stained and fixed as described above.

**Adoptive Transfers.** For total B cell adoptive transfers, RBC-depleted splenocytes were isolated from B6 or B6.NZBc4 mice. The cells were filtered and negatively sorted using a Pan B cell MACs Miltenyi Isolation Column (purity routinely between 90-95%). Eight to ten million sorted B cells were transferred into 8 week old B6.NZBe1.Th1aIgHa recipients. Mice were bled every two weeks until 2 months after injections. For CD5^+ adoptive transfer experiments, between 0.8-1 x 10^6 CD5^+ CD19^+ cells and 8-10 x 10^6 CD5^−CD19^+ B cells were sorted from B6.NZBc4 mice on a FACS ARIA II and injected into 6-8 week old B6.NZBe1 recipients. Mice were sacrificed two months after injection.
Autoantibody Measurements. Anti-chromatin, anti-ssDNA, and anti-dsDNA IgG, IgG1, and IgG2a autoantibodies, were measured by ELISA, as previously described [74]. Briefly, 96 well flat bottom plates were coated with antigen, serum was diluted and added, and bound antibodies were detected using alkaline-phosphatase conjugated anti-IgG, -IgG1, or -IgG2a secondary reagents (Southern Biotech; Birmingham, AL). Substrate (4-nitrophenyl phosphate disodium salt hexahydrate, Sigma-Aldrich) was added, and the OD of each well was measured at a wavelength of 405 nm.

LM Scores. For assessment of kidney damage, kidneys were fixed in formalin, embedded in paraaffin, sectioned (3 μM), and stained with periodic acid-Schiff for light microscopy. All grading was performed by a renal pathologist (G.L.), who was blinded to the strain of origin of the tissue section. The grading scale used for light microscopy was as follows: grade 0, normal glomeruli; grade 1, mesangial expansion, proliferation, or both; grade 2, focal and segmental (endocapillary) proliferative GN; grade 3, diffuse (endocapillary) proliferative GN; grade 4, as 3 but with crescents.

Statistics. For comparisons of differences between three or more groups, a Kruskal-Wallis test was used followed by Dunns’ post-test for multiple comparisons. When only two groups were compared, the Mann-Whiney U test was performed unless otherwise stated. All statistical analyses were performed using GraphPad software (La Jolla, CA, USA).
Results

*iNKT cells do not suppress autoimmunity in B6.NZBc1c4 mice.*

Previous work from our laboratory showed that B6.NZBc1c4 mice exhibit a marked decrease in autoantibodies, reduced kidney damage, and greater survival as compared to B6.NZBc1 mice [74]. These suppressed B6.NZBc1c4 mice retained the same expansion of CD5⁺ B cells and iNKT cells as B6.NZBc4 mice. Given that other groups have shown a regulatory and suppressive role for NKT cells in SLE models, we initially hypothesized that this expansion was acting to protect suppressed mice from fatal autoimmunity.

To evaluate the role of iNKT cells in the observed suppression, B6.NZBc1c4.CD1d-/- mice were generated. Mice that lacked a functional CD1d gene had no NKT (PBS57 Tetramer⁺ TCRβ⁺) cells (Figure 5), whereas heterozygous B6.NZBc1c4.CD1d-/+ mice did not have statistically different frequencies of splenic iNKT cells as compared to CD1d sufficient controls. Notably, the CD1d knockout did not affect the proportion of CD5⁺ B cells (Figure 6). To assess the impact of CD1d gene deletion on the severity of autoimmunity, mice were aged to 8 - 9 months and their autoantibody levels and kidney damage examined. As we had previously observed, the levels of IgG anti-ssDNA and -dsDNA, but not anti-chromatin Abs, were significantly reduced in B6.NZBc1c4 mice as compared to B6.NZBc1 mice, and this resulted predominantly from reduced production of IgG2a auto-Abs. Interestingly, the CD1d
Figure 5: Knockout of NKT cells has no effect on the suppression of fatal autoimmunity in B6.NZBc1c4 mice.

(A) The frequency of NKT cells in the spleens of 8 month old mice. NKT cells were gated as PBS57-CD1d Tetramer$^+$ TCR$\beta^-CD19^-$ and quantified by flow cytometry. (B) Autoantibody levels in the serum of 8-9 month old mice. Antibody levels were measured by antigen-specific ELISA. (C) Light microscopy scores, reflecting the severity of glomerulonephritis, in 8-9 month old mice. Biopsies were scored as outlined in the materials and methods. Each point represents a single mouse, with the lines indicating the arithmetic mean of each group. Statistical analyses were carried out using a Kruskal-Wallis test with select Dunn multiple comparison posttests (A, C) or Mann-Whitney U tests (B). * P < 0.05, ** P < 0.01, ***P <0.001.
Figure 6: Knockout of NKT cells has no impact on the expansion of CD5⁺ B cells or IL-10-producing B cells.

The frequency of splenic (A) CD5⁺ or (B) IL-10-producing B cells in the spleens of 8 month old mice. Cells were stained and quantified by flow cytometry, as outlined in Figure 2. Each point represents a single mouse, with the line for each group representing the arithmetic mean. Statistical analyses were carried out using a Kruskal-Wallis test with select Dunn multiple comparison posttests. * P < 0.05, ** P < 0.01, ***P <0.001.

In contrast to our previous study, there was no reduction in kidney scores seen in 8 month old B6.NZBc1c4 as compared to B6.NZBc1 mice. In our previous study, approximately 30% of the B6.NZBc1c4 mice had reduced numbers of B cells, a phenotype that was not observed in the current study. As these mice had the most attenuated kidney disease, the lack of differences observed in this study may be due to the lack of mice with this phenotype. Nevertheless, as further evidence of a lack of role of NKT cells in disease suppression, the LM kidney scores of B6.NZBc1c4.CD1d⁻/⁻ did not differ significantly from B6.NZBc1c4 CD1d sufficient mice (Figure 5).
knockout had a limited impact on the suppression of auto-Ab production in B6.NZBe1c4 mice (Figure 5). Indeed, B6.NZBe1c4.CD1d−/− mice had significantly lower levels of anti-chromatin IgG and IgG2a autoantibodies when compared to wild-type B6.NZBe1c4 mice, suggesting that NKT cells may be acting to promote rather than suppress autoimmunity in B6.NZBe1c4 mice.

**B6.NZBe4 and B6.NZBe1c4 mice have expansions of IL-10-producing B cells that localize to the CD5⁺ B cell compartment.**

We next examined whether the expanded CD5⁺ B cell population in B6.NZBe4 and B6.NZBe1c4 mice could have a regulatory function. To address this possibility, we began by investigating whether there were increased proportions of IL-10-secreting B cells in these mouse strains. Splenocytes were stimulated with the same combination of LPS, PMA, and Ionomycin that had been used in previous studies to assess Breg numbers/function [289], and the frequencies of IL-10-producing B cells were quantified by flow cytometry (Figure 7). This revealed that B6.NZBe4 and B6.NZBe1c4 mice had a ~1.5-2 fold expansion of splenic IL-10-producing B cells as compared to B6 and B6.NZBe1 mice (Figure 7).

Other groups have shown that Breg cells primarily localize to the CD5⁺ CD1dhigh or the T2-MZP compartments [278]. We therefore examined expression of CD1d and CD5 on the IL-10-producing B cells (Figure 7). In B6.NZBe4 and B6.NZBe1c4 mice, the
Figure 7: B6.NZBc4 and suppressed B6.NZBc1c4 mice have an expansion of IL-10-producing CD5⁺ B cells.

(A) Regions used to gate CD19⁺ cells. Representative results are shown for a 4 month old B6 mouse. (B) Splenocytes from 4 month old mice were stimulated with LPS, PMA, and Ionomycin in the presence of GolgiStop (see materials and methods). Fluorescence minus one (FMO) and IL-10 knockout mice were used to confirm the gating strategy used to identify IL-10-producing B cells. (C) Frequency of splenic IL-10-producing-B cells, gated as above, in 4 month old congenic mice. (D) Representative plots of total CD19⁺ B cells and the IL-10-producing B cell subset from all congenic strains. Frequency of CD5⁺ (E) and CD5⁻ (F) IL-10-producing cells in the spleen, measured as a proportion of total B cells. Positive populations were gated based on quadrants in (D). Frequency of CD5⁺ CD1dhigh (B10) (G) and CD5⁺ CD1dlow (H) IL-10-producing B cells in the spleen. (I) The frequency of IL-10-producing B cells as a proportion of the total splenic CD5⁺ CD19⁻ B cell population. For all IL-10 data, splenocytes were stimulated for 4-5 hours as described in the materials and methods. Each point represents a single mouse, with the lines for each group representing the arithmetic mean. Statistical analyses were carried out using a Kruskal-Wallis test with Dunn multiple comparison posttests. * P < 0.05, ** P < 0.01, ***P <0.001
predominant increase in IL-10-producing cells was found primarily in the expanded CD5⁺ compartment (Figure 7). Within the CD5⁺ compartment, the majority of the expansion of IL-10-producing cells appeared to arise from increases in the CD5⁺ CD1dlow population (Figure 7). Interestingly, the frequency of traditional B10 regulatory cells, which localize to the CD1dhighCD5⁺ B cell compartment, was only slightly increased in B6.NZBc1c4 mice (Figure 7). We also assessed for differences in the levels of IL-10 produced in the gated compartments by examining the MFI for IL-10 staining. To determine whether the increase in IL-10⁺ B cells in B6.NZBc1c4 mice was simply due to an expansion of their splenic CD5⁺ compartment, we measured the frequency of IL-10-producing cells as a proportion of the expanded CD5⁺ pool (Figure 7). Using this measure, we noted that B6.NZBc1c4 mice had a significant expansion of IL-10-producing cells within the CD5⁺ compartment, whereas the proportion of these cells was similar in B6 and B6.NZBc4 mice. These results suggest that CD5⁺ B cells are expanded genetically by a locus on chromosome 4, but further driven to produce IL-10 in the context of the autoimmune environment in B6.NZBc1c4 mice.

**Phenotypic and functional characterization of the expanded IL-10-producing population in B6.NZBc4 mice.**

To better characterize the expanded IL-10-producing pool, we tested the expression of several cell-surface markers that discriminate between various B cell populations. Expression of these markers on IL-10-producing B cells, gated as in Figure 7 was
Figure 8: Phenotypic characterization of IL-10 producing B cells using typical B1a cell markers.

Representative plots of total CD19\(^+\) B cells and the IL-10\(^+\) sub-population stimulated and gated as in Figure 2. Cells were gated based on CD43 and B220 (A), IgM and IgD (B), and CD21 and CD24 expression (C). The frequency of the gated populations as a proportion of total IL-10 producing cells was calculated across congeneric strains (D, E, F, G, & H). Each point represents a single mouse, with the line for each group representing the arithmetic mean. Statistical analyses were carried out using a Kruskal-Wallis test with select Dunn multiple comparison post-tests. * P < 0.05, ** P < 0.01, ***P<0.001.
Figure 9: IL-10 producing plasmablasts are expanded in suppressed mice but represent a rare population in the IL-10⁺ B cell pool.

(A) Representative gating of CD44⁺CD138⁺ plasmablasts from B6 mice. Cells were previously gated on FSC and SSC, with dead cell exclusion. (B) The frequency of plasmablasts across congenic strains as measured by flow cytometry and as gated in A. (C) Representative plots of IL-10 and CD138 expression for the congenic strains. Cells were stimulated and gated as described in Figure 2. (D) The frequency of CD138⁻ IL-10 producing plasmablasts as a proportion of CD138⁺ cells. (E) The frequency of CD138⁻ plasmablasts as a proportion of the IL-10⁺ B cell population. Each point represents a single mouse with the line for each group representing the arithmetic mean. Statistical analyses were carried out using a Kruskal-Wallis test with select Dunn multiple comparison post-tests. * P < 0.05, ** P < 0.01, *** P < 0.001.
compared between strains (Figure 8). Although there was heterogeneity in the cell surface molecules expressed by these cells, a considerable proportion expressed typical B1a makers including higher CD43, low IgD, and low B220 levels. Given the recent identification of suppressive IL-10 producing plasmablasts in mice and humans, we also sought to identify this population in our congenic strains [173,290]. As shown in Figure 9, the plasmablast population was not expanded in 4 month old B6.NZBc1c4 mice. Although there was a CD138 sub-population that produced IL-10 that was significantly expanded in B6.NZBc1c4 mice, this sub-population accounted for only ~1 percent of the total IL-10 B cell population.

Next, we investigated the ability of SLE-related TLR ligands and nuclear antigens to stimulate IL-10 production in CD5+ B cells (Figure 10). Interestingly, CD5+ B cells from both B6 and B6.NZBc4 mice could produce IL-10 when stimulated with a number of TLR agonists and nuclear auto-antigens. Although not significantly different, CD5+ B cells from B6.NZBc4 mice did demonstrate a trend towards higher IL-10 production following stimulation with PolyI:C, histone and chromatin. This finding suggests that the expanded CD5+ population in B6.NZBc4 mice may be more prone to secrete large quantities of IL-10 in response to nuclear auto-antigens, supporting a potential role in the suppression of lupus-like autoimmunity.
Figure 10: CD5⁺ B cells can respond to stimulation with nuclear autoantigens and TLR ligands.

(A&B) The frequency of splenic CD5⁺ and IL-10-producing B cells in the spleens of 4 month old B6 and B6.NZBc4 mice. Splenocytes were stimulated overnight with indicated TLR agonists or nuclear autoantigens, with GolgiStop being added in the last 4 hours of culture. IL-10 production was measured by flow cytometry as previously described in Figure 2. Bars represent the arithmetic mean and error bars the standard error for three to four animals. Statistical analyses were carried out using a Kruskal-Wallis test with select Dunn multiple comparison posttests. * P < 0.05, ** P < 0.01, ***P <0.001.
Figure 11: The expansion of pro-inflammatory T cell subsets is reduced in B6.NZBc1c4 mice and correlates with an increase in IL-10-producing B cells

(A) Representative plot of splenic IFNγ- and IL-17-producing CD4 T cells in 4 month old mice. Cytokine producing CD4 T cells were quantified by flow cytometry after 4.5 hours stimulation with PMA and Ionomycin in the presence of GolgiStop. CD4 T cells were gated first on the basis of FSC and SSC, and then as CD4^+CD19^- Cytokine-producing cells were determined using an FMO. (B, C) The frequency of IFNγ and IL-17 producing CD4 T cells in 4 month old mice. (D, E) Positive correlation between the levels of anti-dsDNA IgG2a antibodies and the frequency of pro-inflammatory IFNγ- and IL-17A-producing CD4 T cells in 8 month old B6.NZBc1c4 mice. (F) The frequency of IL-17A-producing cells is negatively correlated with the expansion of splenic CD5^- B cells in 8 month old B6.NZBc1c4 mice. (G) Kidney light microscopy (LM) scores are negatively correlated with the frequency of IL-10-producing B cells in 8-9 month old B6.NZBc1c4 mice. Statistical analyses were carried out using a Kruskal-Wallis test with select Dunn multiple comparison posttests and linear regression analysis. * P < 0.05, ** P < 0.01, ***P <0.001.
Expansion of regulatory B cells is associated with a reduced frequency of pro-inflammatory CD4 T cells in suppressed B6.NZBc1c4 mice.

We have previously shown that B6.NZBc1 mice have increased proportions of Th1 and Th17 CD4 cells, which appear to play an important role in disease progression. Analysis of splenic frequencies of these two cell subsets revealed that 4 month old B6.NZBc1, but not B6.NZBc1c4 mice had clearly elevated levels of Th1 and Th17 cells (Figure 11). At 8 months of age, there was an inverse correlation between the levels of IL-17A- and IFNγ-producing CD4 T cells and the levels of pathogenic anti-dsDNA IgG2a autoantibodies in B6.NZBc1c4 mice (Figure 11), raising the possibility that inhibition of expansion of these pro-inflammatory subsets limits autoantibody production. In support of a regulatory role for CD5+ B cells in the reduced expansion of pro-inflammatory T cell subsets in B6.NZBc1c4 mice, expansion of CD5+ B cells was negatively correlated with the proportion of IL-17A-producing cells in 8 month old mice (Figure 11). More telling was the finding that the frequency of IL-10-producing B cells negatively correlated to the LM kidney score (Figure 11).

Recent work in other mouse models has shown that regulatory B cells may exert their function by expanding T regulatory cells in a GITR-dependent manner [162]. To address this possibility, the frequency of functional (CD25+Foxp3+) regulatory cells was measured in our mice. At four months of age, there were no differences in the frequency of functional T regulatory cells across all mouse strains (Figure 12). Interestingly, the
Figure 12: Lack of correlation between T regulatory cells and the expansion of CD5⁺ B cells in B6.NZBc1c4 mice.

(A) Representative gating of T regulatory cells in congenic mouse strains. Splenocytes were stained and fixed as described in the materials and methods. (B) The frequency of functional (CD25⁺Foxp3⁺CD4⁺CD3⁺) regulatory cells is unchanged in suppressed B6.NZBc1c4 mice, as measured by flow cytometry. Cells were gated based on FSC, SSC, CD3, CD4, CD25, and Foxp3 expression. (C) The frequency of committed but non-active (CD25⁻Foxp3⁻CD4⁻CD3⁻) regulatory cells is increased in B6.NZBc1 mice with B6.NZBc1c4 mice expressing intermediate levels. (D) The frequencies of functional T regulatory cells and IL-10-producing-cells are not correlated in 4 month old B6.NZBc1c4 mice. Lines for each group represent the arithmetic mean. Statistical analyses were carried out using a Kruskal-Wallis test with select Dunn multiple comparison posttests in A & B, and linear regression analysis in C. * P < 0.05, ** P < 0.01, ***P <0.001.
frequency of CD25+ T regulatory cells was significantly expanded in B6.NZBc1 mice with B6.NZBc1c4 mice showing intermediate levels (Figure 12). This population of cells may represent a reservoir of inactive or non-functional but committed T regulatory cells [291,292]. Thus, the expansion of these cells in B6.NZBc1 mice may represent the byproduct of excessive immune activation, which is reduced in suppressed B6.NZBc1c4. More importantly, when frequencies of IL-10-producing B cells and functional T regulatory cells were compared, there was no correlation between the two groups of regulatory cells, supporting the disassociation of these two populations (Figure 12).

Transfer of B cells from B6.NZBc4 but not B6 mice reduces the frequency of pro-inflammatory T cell populations in autoimmune B6.NZBc1 mice.

To further explore the possibility that the expansion of IL-10-producing CD5+ B cells, associated with the NZB c4 interval, suppresses autoimmunity in B6.NZBc1c4 mice, we used an adoptive transfer approach. 8-10 million total B cells from B6 or B6.NZBc4 mice were transferred into 2 month old autoimmune-prone B6.NZBc1.Thy1+IgHa recipients and their impact on autoantibody levels and expansion of pro-inflammatory T cell subsets assessed 2 months later. As shown in Figure 13, very few of the transferred B cells were still present 2 months following transfer, with no difference observed between transferred B6 and B6.NZBc4 cells in the number of surviving cells. For both transferred B cell populations, the majority of remaining B cells were CD5+ and IL-10-, with slightly higher proportions of transferred B6.NZBc4 cells being CD21hi. Analysis of anti-ssDNA and –dsDNA antibody production in recipient mice revealed generally low levels of
autoantibodies with no significant differences between those that received B6 or B6.NZBc4 B cells (Figure 14). In contrast, there was a clear reduction in the frequency of memory/active CD44hi/CD62L+, and IFNγ- and IL-17A-producing CD4 T cells (Figure 14) in recipient mice that had received B6.NZBc4 as compared to B6 B cells. To investigate whether the CD5+ cells in the transferred B cells were responsible for this decrease, we transferred 1 million cell-sorted CD5+ B cells or 10 million CD5-depleted B cells from B6.NZBc4 mice into B6.NZBc1 recipient mice. These numbers of cells were chosen to mimic the numbers of cells for each population that were present in the total B cell transfers. Following transfer of CD5+ B cells there was a trend toward decreased frequencies of memory/active and IFNγ producing CD4 T cells but not IL-17A producing CD4 T cells, as compared to mice receiving CD5- B cells (Figure 14).
Figure 13: Adoptively transferred B cells from B6 and B6.NZBc4 mice can be identified in host autoimmune animals but do not express CD5.

10 million total B cells from B6 or B6.NZBc4 mice were transferred into B6.NZBc1.Thy1aIgHa recipients and their survival measured after 2 months. (A) Representative regions used for gating of transferred IgMb+IgMa- cells. (B) Scatter plot showing pooled results from multiple transfers. (C) Representative dot plots of CD21 and CD5 expression on B cells gated as outlined in Figure 2. Transferred (IgMb+IgMa- CD19+, bold) B cells are overlaid on total CD19+ (gray) B cells. (D, E, F) The proportion of transferred IgMb+IgMa-CD19+ cells that are CD5+, CD21+, or IL-10+, respectively. (G) The frequency of splenic NKT cells (PBS57-CD1d Tetramer+ TCRβ+CD19+) is unchanged in recipient mice. (H) The frequency of splenic IL-10-producing B cells, gated as in Figure 2, is unaltered in recipient mice. Each point represents a single mouse with lines representing the arithmetic mean. Statistical analyses were carried out using a Kruskal-Wallis test with select Dunn multiple comparison posttests or Mann-Whitney U tests. * P < 0.05, ** P < 0.01, ***P <0.001.
Figure 14: Transfer of total B cells from B6.NZBc4 mice reduces the frequencies of pro-inflammatory IL-17A- and IFN-γ-producing CD4 T cells but has no impact on autoantibody levels.

B6 or B6.NZBc4 total B cells were transferred into B6.NZBc1 recipient mice and disease severity assessed after two months. Control animals received injection with equal volumes PBS. (A) Levels of anti-ssDNA and dsDNA autoantibodies, as measured by ELISA, remained unchanged in all recipients. (B) Representative plot of memory/active CD44+CD62L- CD4 T cells from PBS control-injected mice. (C) Reduced frequency of splenic activated/memory CD44+ CD4+ T cells, as measured by flow cytometry, in recipients that received total B6.NZBc4 B cells when compared to B6 transferred controls. (D) Representative plot of IFNγ and IL-17 producing CD4 T cells in recipient mice. Cells were stimulated and gated as described in the materials and methods. The frequency of IFNγ- (E) and IL-17A- (F) producing CD4 T cells measured after 4.5 hours stimulation with PMA and ionomycin in the presence of GolgiStop. (G, H, & I) 1 million CD5+ CD19+ and 10 million CD5-CD19+ B cells were sorted from B6.NZBc4 mice and injected into B6.NZBc1 recipients. The frequency of activated/memory CD44+CD62L- (F), and pro-inflammatory IFNγ-producing (G) and IL-17A-producing (H) CD4 T cells was measured in recipients by flow cytometry as above. Each point represents a single mouse, with lines representing the arithmetic mean for each group. Statistical analyses were carried out using a Kruskal-Wallis test with select Dunn multiple comparison posttests or Mann-Whitney U tests. * P < 0.05, ** P < 0.01, ***P <0.001.
Discussion

In this study, we have shown that the expansion of IL-10-producing CD5+ B cells in B6.NZBc1c4 mice is associated with protection against spontaneous lupus-like autoimmunity and decreased frequencies of pro-inflammatory Th1 and Th17 cells. Consistent with the possibility that IL-10-producing CD5+ cells limit expansion of pro-inflammatory T cell subsets in these mice, adoptive transfer of B cells from B6.NZBc4 mice into autoimmune B6.NZBc1 recipients lowered the frequency of Th1 and Th17 cells. Nevertheless, studies in aged B6.NZBc1c4 mice showed a clear inverse correlation between the frequencies of CD5+ or IL-10-producing B cells and the frequency of pro-inflammatory T cell populations and disease severity, suggesting that these cells may also act to suppress autoantibody production. To our knowledge, this is the first time that an expansion of regulatory CD5+ B cells has been identified in a spontaneous mouse model of SLE.

Given the increase in splenic NKT cell frequencies observed in suppressed B6.NZBc1c4 mice, we investigated their relative role in disease progression through generation of CD1d knockouts that lack NKT cells. Although NKT cells can act to rapidly secrete large quantities of cytokine and have been shown in multiple autoimmune models to modulate the autoimmune response, their absence had little or no impact on the severity/progression of autoimmune disease in our mice. This finding suggests that genetically-derived concurrent immune defects may override the relative importance of NKT cells in our model. In support of this concept, NKT cells from B6.NZBc1c4 mice
demonstrate impaired function following stimulation with the prototypic ligand, αGalCer (unpublished observation), which we have localized to the parental B6.NZBc1 strain. Thus, the lack of effect of NKT cell deletion on the disease course in our mice, despite the expansion of this population, may reflect the fact that NKT cell function is already impaired. Importantly, the loss of NKT cells had no impact on the expansion of CD5⁺ B cells or the proportion of IL-10-producing B cells in our mice, and clearly did not abrogate the suppression seen in these mice.

Recent literature has provided evidence for a heterogeneous population of CD5⁺ IL-10-producing B cells capable of controlling various autoimmune responses [166]. In our mice, we found an expansion of this population that localized to chromosome 4. Contrary to the established literature, the expansion of CD5⁺ IL-10-producing B cells in our mice was predominantly located within the CD1d<sub>low</sub> compartment rather than the CD1d<sub>high</sub> compartment of typical B10 cells. Using various stimuli, we showed that splenic CD5⁺ B cells could secrete IL-10 in response to a number of SLE-related TLR ligands and nuclear auto-antigens. This finding may explain the capacity of this population to suppress disease in lupus-prone B6.NZBc1c4 mice, where the autoimmune response is specifically focused upon nuclear antigens. Notably, the proportion of IL-10-producing CD5⁺ B cells was increased in B6.NZBc1c4 as compared to B6.NZBc4 mice, raising the possibility that these cells are being recruited or activated in response to the autoimmune processes initiated by the NZB chromosome 1 interval. Similar correlations have been shown in SLE patients, with increased levels of circulating regulatory B cells.
reported during disease flares [293]. Taken together, these findings suggest that stochastic expansion or generation of CD5+ B cells may occur through stimulation with auto-antigens.

Several other groups have produced and/or characterized mice with NZB chromosome 4 intervals. An overlapping interval from New Zealand Mixed (NZM) mice, comprised of NZB-derived genetic material, has been shown to promote the expansion of splenic and peritoneal B1a cells. However, in contrast to our results, these studies characterized this expansion as pro-inflammatory [294,295]. The origin of this difference likely arises from the difference in the length of the NZB interval between the NZM-derived congenic interval and the NZB interval in our mice, which contains an additional, more centromeric NZB genetic region. This proposition is supported by the observation that groups working on NZM-derived chromosome 4 congenic mice have never reported an expansion of NKT cells or an increase in IL-10-producing B cells [294]. Thus, our larger chromosome 4 congenic interval, which is purely NZB-derived, may contain additional genetic defects that separately drive either the expansion of NKT cells or the production of IL-10-secreting B cells, and which are responsible for the suppression observed in our bicongenic mice. In unpublished observations, we have narrowed both of these expansions to an NZB-derived region spanning 91 to 123Mb of chromosome 4. Notably, within this interval is a second NZB-derived genetic locus that has been shown to lead to reduced receptor editing of DNA-reactive B cells [87]. It is possible that this
locus leads to an increased proportion of nuclear-antigen reactive B1 cells in B6.NZBc4 mice that could enhance their ability to regulate lupus-like autoimmunity.

The expansion of pro-inflammatory Th1 and Th17 cells has been well documented to play a significant role in SLE pathogenesis[109,115,296]. Studies have shown that these cell types support autoantibody production and can directly cause damage by infiltrating into kidneys[297]. In our bicongenic B6.NZBc1c4 mice, at 8 months of age the expansion of CD5+ B cells negatively correlated with the frequency of IL-17A-producing CD4 T cells and kidney damage. This finding suggests that CD5+ IL-10-producing cells may act to prevent the expansion of pro-inflammatory T cell subsets and thereby limit autoantibody generation and kidney disease.

To validate the suppressive role of the CD5+ expansion in B6.NZBc1c4 mice, we transferred total and sorted CD5+ B cells into autoimmune-prone mice. Although we could not detect any changes in autoantibody levels or kidney damage, we did notice a striking decrease in the levels of memory/activated, Th1, and Th17 CD4 T cell subsets upon transfer of total B cells from B6.NZBc4 mice. Transfer of cell sorted CD5+ B cells from B6.NZBc4 mice resulted in a similar trend for memory/activated and Th1 but not Th17 CD4 T cells. The absence of differences in autoantibody titres or kidney damage after transfer of total B cells may reflect a need to perform multiple injections. From our survival data, it is clear that at 2 months post-injection, transferred CD5+ B cells can no longer be identified in the spleen of recipient mice (Figure 13). Simply put, at this time
point the cells may be exhausted or depleted and unable to continue to control autoimmunity. Other groups have shown similar findings with GFP transgenic mice, showing that regulatory B cells only transiently express IL-10 [298]. An alternative possibility is that the mice did not have enough time to produce sufficiently high autoantibody levels to detect differences.

Although we did not directly examine the mechanisms by which the B6.NZNe4 B cells prevent expansion of pro-inflammatory T cell subsets and regulate autoimmunity, it is unlikely that this occurs as the result of expansion of regulatory T cells, as has been suggested by some groups [162]. The proportion and number of T regulatory cells were unaffected by the expansion of IL-10-producing CD5+ cells in B6.NZBc1c4 mice, which had lower levels of Foxp3-expressing cells when compared to parental B6.NZBc1 controls. We were not able to address whether IL-10 secretion plays a role in limiting pro-inflammatory T cell expansion in recipient mice, because knockout of IL-10 in B6.NZBc4 mice affected not only IL-10 secretion, but also normalized the CD5+ B cell expansion (unpublished observations).

In summary, we conclude that the expanded NZB chromosome 4-derived CD5+ B cell population is not pro-inflammatory, but instead likely has a regulatory phenotype. Activation of this population in an autoimmune context may lead to suppression of autoimmunity by inhibiting pro-inflammatory T cell subsets that play an important role in the production of pathogenic anti-nuclear antibodies.
CHAPTER III

IL-10 Production is Critical for Sustaining the Expansion of CD5+ B and NKT cells and Restraining Autoantibody Production in Congenic Lupus-Prone Mice.

Yuriy Baglaenko¹,², Kieran Patricia Manion¹,², Nan-Hua Chang¹, Eric Gracey¹,², Christina Loh¹,²,³, Joan E. Wither¹,²,⁴

¹ Krembil Research Institute, University Health Network, Toronto, Ontario, Canada; ² Department of Immunology, University of Toronto, Toronto, Ontario, Canada; ³ Department of Microbiology and Immunology, Stanford University, Stanford, California, USA (Current Address of CL); ⁴ Department of Medicine, University of Toronto, Toronto, Ontario, Canada

(All experiments were performed by Y. Baglaenko; all other authors assisted with experiments or helped with experimental design and analysis)

Published in *PLoS One*

July 2015, pp. 311-20.

© Baglaenko et al.
Abstract

The development and progression of systemic lupus erythematosus is mediated by the complex interaction of genetic and environmental factors. To decipher the genetics that contribute to pathogenesis and the production of pathogenic autoantibodies, our lab has focused on the generation of congenic lupus-prone mice derived from the New Zealand Black (NZB) strain. Previous work has shown that an NZB-derived chromosome 4 interval spanning 32 to 151 Mb led to expansion of CD5+ B and Natural Killer T (NKT) cells, and could suppress autoimmunity when crossed with a lupus-prone mouse strain. Subsequently, it was shown that CD5+ B cells but not NKT cells derived from these mice could suppress the development of pro-inflammatory T cells. In this paper, we aimed to further resolve the genetics that leads to expansion of these two innate-like populations through the creation of additional sub-congenic mice and to characterize the role of IL-10 in the suppression of autoimmunity through the generation of IL-10 knockout mice. We show that expansion of CD5+ B cells and NKT cells localizes to a chromosome 4 interval spanning 91 to 123 Mb, which is distinct from the region that mediates the majority of the suppressive phenotype. We also demonstrate that IL-10 is critical to the restraining autoantibody production and surprisingly plays a vital role in supporting the expansion of innate-like populations.


Introduction

Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disorder characterized by the production of pathogenic anti-nuclear antibodies (ANAs). A combination of genetic and environmental factors interacts to initiate and exacerbate disease in patients with SLE. To decipher the genetics of SLE initiation and progression, studies in our lab and others have focused on generating congenic mice where susceptibility or suppressor loci from lupus-prone mouse strains can be examined in isolation [299].

The prototypic murine model of SLE is the F1 cross between the New Zealand Black and New Zealand White (NZB/W F1) mouse strains, which develop high titer ANAs and fatal renal disease by 8 months of age. Since NZB/W F1 mice have a mixed genetic background, homozygous derivatives were created to map the genetic defects associated with disease. One of these derivatives, the NZM2410 mouse strain, was used to identify three major susceptibility loci on chromosomes 1, 4, and 7 named Sle1, Sle2, and Sle3, respectively [53,58,300]. Although the Sle1 and Sle3 susceptibility loci were derived from the NZW parent, Sle2 contained a mixture of NZB and NZW genetic material, with the NZB interval extending from 100 to 128 Mb.

Studies from our lab have focused on investigating how New Zealand Black (NZB) genes on chromosomes (c) 1, 4, and 13 influence immune function. Initial work on B6 mice with an introgressed NZB c4 interval extending from 32 to 151 Mb, denoted
B6.NZBc4, identified an expansion of two innate-like populations, B1a cells and Natural Killer T cells (NKT), in the absence of autoantibody production or renal disease [72]. As previous mapping studies had suggested the presence of a lupus-susceptibility gene within this interval, we anticipated that crossing this interval onto the lupus-prone B6.NZBc1 congenic background would lead to augmented autoimmune disease. However, this cross resulted in suppression of disease with reduced autoantibody levels and kidney damage as compared to mice with the NZB c1 interval alone [74].

In a recent follow-up publication, we investigated the immune mechanism leading to this suppression and ruled out a regulatory role for the expanded NKT cell population by creating CD1d knockout B6.NZBc1c4 bicongenic mice. Instead, a possible regulatory role for the expanded splenic CD5⁺ B cell compartment was identified [301]. Given the recent interest in regulatory B cells, we hypothesized that IL-10 production by CD5⁺ B cells was critical to suppression in our lupus-prone mice.

Over the last decade, research has highlighted the suppressive role of IL-10 producing regulatory B cells in various autoimmune models ranging from collagen-induced arthritis to experimental autoimmune encephalomyelitis [166,276,302]. Pertinent to our studies, IL-10 producing regulatory B cells have also been identified to play a suppressive role in several mouse models of SLE [180,181,284]. In the NZB/W F1 model, depletion of B cells early in disease resulted in a loss of regulatory B cells and an accelerated phenotype [180]. In the MRL/lpr mice model, which have a defect in Fas and
are therefore prone to autoimmunity, induction of regulatory B cells through anti-CD40 stimulation and subsequent adoptive transfer was shown to have an IL-10 dependent protective effect [183]. Disease modulating IL-10-producing B cells have been characterized in numerous B cell compartments ranging from typical B1 and marginal zone (MZ) B cells to specific sub-populations such as transitional 2-marginal zone precursors and CD1dhiCD5+ B10 cells [166,275]. Although their ontogeny and phenotypic characteristics are still not entirely known, through use of knockout animals and blocking antibodies, IL-10 has been shown to play a central role in the suppressive function of these cells [158,276].

IL-10 is a pleiotropic cytokine produced by a number of leukocyte populations that impacts on immune regulation and tissue homeostasis [189,279]. While its expression by regulatory B cell populations suggests that it may play a predominantly suppressive role in SLE, the evidence supporting this is contradictory. Studies of murine models of lupus have identified both pathogenic and suppressive roles for IL-10 in disease. In the NZB/W F1 mouse model, administration of blocking IL-10 antibodies reduced disease severity while prolonged treatment with recombinant IL-10 accelerated disease [195]. In contrast to these studies, knockout of IL-10 exacerbated disease and administration of recombinant protein lowered the levels of autoantibodies in MRL/lpr mice [303]. However, a B cell specific IL-10 knockout bred onto the MRL/lpr background had no effect on the progression or severity of SLE [182]. In support of a suppressive role for IL-10 in SLE, triple-congenic B6.Sle1.Sle2.Sle3 mice that were
forced to produce low levels of IL-10 by introduction of a viral vector had delayed autoantibody production and decreased nephritis [196].

In this study, we have used a combination of NZB c4 sub-congenic mouse strains and IL-10 knockout mice to further investigate the immune mechanisms leading to suppression of autoimmune disease and expansion of CD5+ B cells and NKT cells in B6.NZBc4 mice. We show that although suppression of autoimmunity and expansion of these cell subsets localize to different regions on NZB c4, both are dependent upon IL-10. To our knowledge, this is the first study to identify a possible link between global IL-10 production and the survival and/or expansion of CD5+ B cells and NKT cells, suggesting a possible role for IL-10 in supporting suppressive immune populations.
Materials and Methods

**Ethics Statement.** Mice were housed in a Canadian Council on Animal Care approved facility at the Krembil Research Institute in the Krembil Discovery Tower, part of the University Health Network. All experiments performed in this study were approved by the Animal Care Committee of the University Health Network (Animal Use Protocol 123).

**Mice.** B6 and NZB mice were purchased from Taconic (Germantown, NY) and Harlan Sprague Dawley (Blackthorn, England), respectively. Congenic mice were generated as previously described [74]. B6.IL-10-/- (B6.129P2-Il10tm1Cgn/J) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and bred onto the various congenic backgrounds using primer assisted breeding. Only female mice were used for experiments in this study, with littermate controls.

**Flow Cytometry.** Splenocytes were harvested and RBC lysed, as previously described [74]. Briefly, half a million cells were incubated with mouse IgG (Sigma-Aldrich, St Louis, MO, USA) for 15 minutes on ice prior to staining with various combinations of directly conjugated mAbs for 30 minutes on ice. The following antibodies were used for primary staining (all purchased from BioLegend, San Diego, CA, USA or BD Biosciences, San Diego, CA, USA): FITC-conjugated anti-TCRβ(H57-597), -CD3ε(145-2C11), -CD23(B3B4); PE-conjugated anti-NK1.1(PK136), -CD24(M1/69), -CD5(53-7.3), -B220(RA3-6B2); PE-Cy7 anti-CD19(6D5), and -CD8(53-6.7); Allophycocyanin-conjugated anti-CD21(7E9), -CD19(6D5), -CD5(53-7.3), and -CD25(3C7); BV605 conjugated anti-B220(RA3-6B2) and -CD3ε(145-2C11); and Pacific Blue-conjugated
anti-CD4(GK1.5) and -B220(RA3-6B2). Dead cells were identified by staining with 0.6µg/mL Propidium Iodide (Sigma Aldrich, St Louis, MO, USA). Allophycocyanin-conjugated unloaded and PBS-57–loaded mouse CD1d tetramers were generously provided by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). Events were collected on a BD LSRII or BD FACSCanto and analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA).

Detection of IL-10-producing B cells. IL-10 production by B cells was examined as previously described [176]. Briefly, 0.5 x 10^6 RBC-depleted splenocytes were plated in 96 well flat bottom plates and stimulated for 4-5 hours with PMA (50ng/mL, Sigma-Aldrich, St Louis, MO, USA), Ionomycin (500ng/mL) Sigma-Aldrich, St Louis, MO, USA), and LPS (10µg/mL, Sigma Aldrich, E coli 011:B4, St Louis, MO, USA) in the presence of GolgiStop (BD Biosciences, San Diego, CA, USA). Following stimulation, cells were stained with Near Infrared Live/Dead stain (Gibco, Waltham, MA, USA) and various conjugated antibodies directed against extracellular markers, and then fixed and permeabilized. The cells were then stained with allophycocyanin-conjugated anti-IL-10 (JES5-16E3, BioLegend, San Diego, CA, USA) for 30 minutes on ice.

Autoantibody Measurements. Anti-chromatin, anti-ssDNA, and anti-dsDNA IgM, IgG, IgG1, and IgG2a autoantibodies, were measured by ELISA, as previously described [74]. Briefly, 96 well flat bottom plates were coated with antigen and left overnight. Serum was diluted 1 in 50 and added in triplicate to the plates. Bound antibodies were detected using alkaline-phosphatase conjugated anti-IgM, -IgG, -IgG1, -IgG2a, IgG2b, IgG2c, or IgG3 secondary reagents (Southern Biotech; Birmingham, AL). Substrate (4-nitrophenyl
phosphate disodium salt hexahydrate, Sigma-Aldrich, St Louis, MO, USA) was added, and the OD of each well was measured at a wavelength of 405 nm. Values were standardized from plate to plate by running known B6 and NZB controls.

**CFSE Staining and Peritoneal B cell Culture.** Peritoneal lavages were taken from mice previously injected with 5mL complete RPMI 1640 (Gibco, Waltham, MA, USA) media supplemented with 10% FBS (Wisent, ST-BRUNO, Quebec, Canada), L-glutamine (Gibco, Waltham, MA, USA), non-essential amino acids (Gibco, Waltham, MA, USA), and penicillin and streptomycin (Gibco, Waltham, MA, USA). Immediately after lavage, cells were transferred to T25 flasks (BD Biosciences, San Diego, CA, USA) and cultured for 2 hours in a 37°C, 5% CO₂ tissue culture incubator. Suspension cells were collected and washed with PBS prior to staining with CFSE (Gibco, Waltham, MA, USA). 10⁵ stained cells were left unstimulated in 96 well flat bottom culture plates (BD Biosciences, San Diego, CA, USA). After 5 days, cells were collected and stained as described above.

**Statistics.** For comparisons of differences between three or more groups, a Kruskal-Wallis test was used followed by Dunns’ post-test for multiple comparisons. For comparison between wild-type and knockout animals, Mann-Whitney U tests were performed. All statistical analyses were performed using GraphPad software (La Jolla, CA, USA).
Results

*The expansion of innate-like lymphocytes localizes to the New Zealand Black chromosome 4 interval spanning 91 to 123 Mb.*

As outlined previously, introgression of an NZB c4 interval spanning 32 to 151 Mb onto the B6 genetic background led to an increase in CD5⁺ B cells and NKT cells [72]. In an effort to better characterize and delineate the genes involved in these expansions and disease suppression, sub-congenic mice were bred with shorter NZB c4 intervals (Figure 15). Innate-like immune populations were then characterized by flow cytometry within the peritoneal and splenic compartments of 4 month old full-length and sub-congeneric B6.NZBc4 mice. As reported previously [72], B6.NZBc4 mice have a slight expansion of total peritoneal cells, but not total splenocytes (Figure 16).

In sub-congenic mice, expansion of splenic CD5⁺ B cells, peritoneal B1a cells, and splenic NKT cells (Figure 15) was only seen in mice with the middle sub-congenic interval B6.NZc4(91-123), termed B6.NZBe4m for simplicity, which largely recapitulated the expansion seen for the full-length B6.NZBc4 mice. Although there was a trend to decreased levels of peritoneal B1a cells in B6.NZBe4m as compared to B6.NZBc4 mice, this did not achieve statistical significance. However, we cannot rule out a contribution of genes in the telomeric B6.NZBc4(123-151) interval, henceforth termed B6.NZBt, to this phenotype as we have previously noted minor increases in the peritoneal B1a cell population in these mice. Surprisingly, the increase of NK1.1⁺ cells...
A

B

C

D

E
Figure 15: The expansion of peritoneal B1a B cells, splenic CD5+ B cells, and NKT cells localizes to an NZB-derived interval spanning 91 to 123 Mb on chromosome 4.

(A) Figure illustrating the NZB chromosome 4 congenic strains used in these studies. D4Mit markers demarcate the known boundaries of introgression. Splenic and peritoneal immune cell frequencies were measured by flow cytometry from 4 month old mice. (B) Splenic CD5+ B cells were measured based on FSC, SSC, CD19, and CD5 staining. (C) Frequencies of peritoneal cells were identified by granularity and size and gated as CD19+ and CD5+. (D) Splenic NKT cells were gated based on size and granularity using FSC, SSC, and gated as PBS57 Tetramer+ NK1.1-. (E) Splenic NK cells were measured based on FSC, SSC, and gated as NK1.1+ and PBS57 Tetramer-. Each point represents a single mouse, with the lines indicating the median of each group. Statistical analyses were carried out using a Kruskal-Wallis test with select Dunn multiple comparison posttests to control B6 mice. * P < 0.05, ** P < 0.01, ***P <0.001.
Figure 16: Total spleen and peritoneal cavity counts.
Total cell numbers for peritoneal and splenic cells were counted using a hemocytometer. Total cell counts for mice used in Figure 1(A,B), Figure 2(C,D) and Figures 3-6(E,F). Each point represents a single mouse, with the lines for each group representing the median. Statistical analyses were carried out using a Mann-Whitney U tests.
(Figure 15) seen in B6.NZBc4 mice could not be recapitulated with any single NZB interval, suggesting that a combination of genes is required to promote this phenotype.

The expansion of B1a and NKT cells has minimal impact on the suppression of autoimmunity in B6.NZBc1c4t mice.

We have previously shown that crossing the full-length (32-151 Mb) NZB c4 interval onto the lupus-prone B6.NZBc1 background leads to suppression of autoantibody production and renal disease, and have provided evidence through adoptive transfer experiments that this was mediated by a suppressive effect of NZB c4 CD5⁺ B cells [74,301]. This led us to hypothesize that the suppression was the result of the expansion of CD5⁺ B cells in B6.NZBc4 mice. To address this hypothesis, bicongenic mice were generated with the telomeric NZB c4 interval spanning 123 to 151 Mb (c4t) and contrasted to bicongenic mice with the full-length NZB c4 interval. Mice were aged to 8 months of age and analyzed for autoantibody production by antigen-specific ELISA. In contrast to bicongenic mice with the full-length interval (B6.NZBc1c4), bicongenic mice with the telomeric interval (B6.NZBc1c4t) had minimal, non-significant increases in the proportions of splenic CD5⁺ B cells, NKT cells, and IL-10-producing B cells as compared to B6.NZBc1 mice (Figure 17). Although we observed a trend (p=0.0559) towards increased mortality in B6.NZBc14t mice suggesting a slight decrease in suppression (Figure 17), production of IgM anti-dsDNA and –chromatin, together with IgG anti-ssDNA and –dsDNA antibodies was equivalently suppressed in both bicongenic
Figure 17: Suppression of anti-ssDNA and –dsDNA autoantibody production in bicongenic B6.NZBe1c4(123-151) mice in the absence of either CD5⁺ B cells or splenic NKT cell expansion.

Frequencies of splenic CD5⁺ B cells (A), NKT cells (B), and IL-10 competent B cells (C) were measured by flow cytometry in 8 to 10 month old mice. (D) Survival curves of aged B6.NZBe1c4 and B6.NZBe1c4t mice show a trend (p=0.0559) towards increased death in B6.NZBe1c4t mice. Levels of anti-ssDNA, –dsDNA, and –chromatin IgM, IgG and IgG2a (E) were measured by antigen-specific ELISA. To define the high levels of anti-chromatin autoantibodies in B6.NZBe1c4(123-151) mice, IgG2b, IgG2b, and IgG3 anti-chromatin antibodies were measured by ELISA (F). Each point represents a single mouse, with the lines for each group representing the median. Statistical analyses were carried out using a Kruskal-Wallis test with Dunn multiple comparison posttests between all groups. * P < 0.05, ** P < 0.01, ***P < 0.001.
mouse strains as compared to B6.NZBc1 mice (Figure 17). However, some small differences in the ability of the two intervals to suppress IgG anti-chromatin antibody production were seen. There was a trend to decreased suppression of IgG anti-chromatin antibody production with the telomeric NZB c4 interval, particularly for the IgG2b and IgG2c subclasses (Figure 17). Thus, the genetic locus mediating suppression of autoimmunity in bicongenic mice is distinct from that leading to the marked expansion of CD5+ and NKT cells on NZB c4, with the exception of a minor additive effect on IgG anti-chromatin antibody production.

Knockout of IL-10 is sufficient to breach tolerance in B6.NZBc4 mice

Previous mapping studies suggested the presence of a lupus-susceptibility locus on NZB c4, however B6.NZBc4 mice do not produce anti-nuclear autoantibodies [72]. Since we had previously shown that the CD5+ B cells in B6.NZBc4 mice have a regulatory function and produce IL-10, we questioned whether knockout of IL-10 could uncover autoimmunity [301]. Knockout mice were produced by backcrossing an IL-10 gene deletion onto both the B6.NZBc4 and B6.NZBc4m backgrounds, with the efficacy of the knockout being confirmed by measuring IL-10 production after stimulation with LPS, PMA, and ionomycin in the presence of monensin (Figure 18).

Supporting our previous findings suggesting that CD5+ IL-10-producing B cells are suppressive, IL-10 knockout resulted in increased production of anti-ssDNA, -dsDNA, and -chromatin IgM autoantibodies in B6.NZBc4 mice, as measured by antigen-
Figure 18: Knockout of IL-10 is penetrant in B6, B6.NZBc4m, and B6.NZBc4 mice. Representative flow cytometry plot and results of IL-10 knockout in congenic animals. Splenocytes were stimulated for 4-5 hours with LPS, PMA, and Ionomycin in the presence of GolgiStop. IL-10 knockout was penetrant in all animals with a complete loss of cytokine production. Each point represents a single mouse, with the lines for each group representing the median.
Figure 19: Knockout of IL-10 in B6.NZBc4 but not B6.NZBc4m mice results in a breach of tolerance to ssDNA, dsDNA, and chromatin.

Levels of anti-ssDNA, -dsDNA, and –chromatin antibodies were measured in 4 month old mice by ELISA. Each point represents a single mouse, with the lines for each group representing the median. Statistical analyses were carried out using a Mann-Whitney U test between homozygous and IL-10 knockout animals of the same genetic background. * P < 0.05, ** P < 0.01.
Figure 20: Levels of IgG1 and IgG2a autoantibodies in IL-10 knockout mice are unchanged.
Levels of anti-ssDNA, -dsDNA, and –chromatin were measured as previously described. Each point represents a single mouse, with the lines for each group representing the median.
specific ELISA on serum from 4 month old mice (Figure 19). B6.IL-10-/- mice also exhibited an increase in IgM anti-ssDNA autoantibodies, but not other nuclear antigens. Both B6.NZBc4m and B6.NZBc4 mice had increased levels of anti-dsDNA IgG autoantibodies (Figure 19). However, there were no changes in the levels of IgG1 or IgG2a autoantibodies (Figure 20), suggesting a largely T cell independent breach of tolerance. This finding is in line with our other observations, which have shown that T and dendritic cell defects from genetic loci on NZB c1 are required to promote SLE and IgG anti-nuclear antibody production in the NZB mouse strain [68]. From this data, it is evident that only B6.NZBc4 mice had a consistent breach in autoantibody production, suggesting the presence of additional NZB-derived c4 loci that can exacerbate autoimmunity.

*Expansion of splenic CD5+ B cells and peritoneal B1a cells is reliant on IL-10.*

Since knockout of IL-10 led to enhanced autoantibody production, we sought to determine whether this resulted solely from a lack of IL-10 production or whether other immunologic changes might contribute to this breach of tolerance. As we had previously shown that adoptive transfer of NZBc4 CD5+ B cells led to enhanced suppression of T cell pro-inflammatory cytokine production in B6.NZBc1 autoimmune mice, as compared to transfer of CD5- cells, we examined whether introduction of the IL-10 knockout onto the B6.NZBc4 background affected this population [301].
As shown in Figure 21(panels A and B), knockout of IL-10 significantly reduced the frequency of CD5⁺ B cells in the spleen and peritoneum of both control and NZBc4 congenic mouse strains. However, in the IL-10 knockout NZBc4 mouse strains, the proportion of these cells remained somewhat expanded as compared to that seen in B6 IL-10 knockout mice.

The loss of these two CD5⁺ populations prompted us to investigate whether expansion of other innate-like immune populations was also altered in our congenic IL-10 knockout mice. As has been previously shown by others, since proliferation of NK cells is reliant on IL-10, knockout of this cytokine significantly reduced the proportion of these cells in all mouse strains examined (Figure 21) [304,305]. Surprisingly and previously unreported, the expansion of NKT cells was also dependent on IL-10 and was significantly reduced in B6 and B6.NZBc4m mice (Fig 4D).

Given the relationship between regulatory CD5⁺ B cells and T regulatory cells [162], we also assessed the frequency of these cells in B6.NZBc4m mice. However, the loss of CD5⁺ B cells had no impact on T regulatory cells in IL-10 knockout B6.NZBc4m mice (Figure 22).

Finally, we assessed whether the IL-10 knockout affected the frequency of splenic B cell populations. Importantly, IL-10 did not alter the number of splenocytes or
Figure 21: The expansion of CD5^+ B cells, NK and NKT cells is impacted by the loss of IL-10.

The frequency of splenic and peritoneal cells was measured in 4 month old mice by flow cytometry. Representative gating and results for splenic CD5^+ B cell (A), peritoneal CD5^+ B cell (B), NK cell (C) and NKT cell frequencies (D). Each point represents a single mouse, with the lines for each group representing the median. Statistical analyses were carried out using a Mann-Whitney U test between homozygous and IL-10 knockout animals of the same genetic background. * P < 0.05, ** P < 0.01, ***P <0.001.
**Figure 22:** The frequency of splenic regulatory T cells is unchanged in B6.NZBc4m IL-10 knockout mice.

(A) Representative flow cytometry plot of CD25⁺Foxp3⁺ and Foxp3⁺ T regulatory cells from 4 month old B6 mice. (B) The frequency of splenic Foxp3⁺ T cells is unchanged in IL-10 knockout mice. (C) The frequency of CD25⁺ T regulatory cells was significantly increased in B6 IL-10 knockout mice but unchanged in congenic B6.NZBc4m mice regardless of IL-10 status. Each point represents a single mouse, with the lines for each group representing the median. Statistical analyses were carried out using a Mann-Whitney U test between homozygous and IL-10 knockout animals of the same genetic background. * P < 0.05, ** P < 0.01.
Figure 23: Knockout of IL-10 in full-length B6.NZBc4 but not B6.NZBc4m mice resulted in a loss of transitional B cells and an expansion of marginal zone B cells.

(A) Representative gating of transitional (CD21<sub>lo</sub>CD23<sup>-</sup>), follicular (CD21<sub>int</sub>CD23<sup>+</sup>), and marginal zone/marginal zone precursor (CD21<sub>hi</sub>CD23<sup>-</sup>) B cells from 4 month old mice. Frequencies of splenic B cell subsets were measured by flow cytometry. (B,C,D) The frequency of follicular, transitional and marginal zone B cells, respectively, was measured by flow cytometry as gated in (A). Each point represents a single mouse, with the lines for each group representing the median. Statistical analyses were carried out using a Mann-Whitney U test between homozygous and IL-10 knockout animals of the same genetic background. * P < 0.05, ** P < 0.01, ***P < 0.001.
proportion of B cells between mouse strains (Figure 23). As shown in Figure 24, there was a significantly reduced frequency of transitional B cells (CD21^{lo}CD23^{lo}), and expanded proportion of marginal zone B cells (CD21^{hi}CD23^{-}) in B6.NZBc4 IL-10 knockout mice. In contrast, the frequency of follicular B cells (CD21^{int}CD23^{high}) was unaffected in any of the mouse strains. This data raises the possibility that an increase in marginal zone B cells may contribute to the production of IgM autoantibodies in B6 and B6.NZBc4 mice, as other groups have demonstrated that marginal zone B cells can produce IgM in the absence of T cell help [90].

**Production of IL-10 is critical to peritoneal B cell survival**

Previously published work has localized the expansion of peritoneal CD5^{+} B cells in NZM2410 mice to a region overlapping with the NZBc4m interval and shown that this is due to defective p18 expression [294,306]. Loss of p18, a cell cycle inhibitor, results in increased turnover of peritoneal B1a cells.

Thus, to further explore the underlying mechanism leading to the reduction in CD5^{+} B cells in IL-10 knockout B6 and NZB c4m congenic mice, resting peritoneal lavage cells from B6 and B6.NZBc4m mice were stained with CFSE and cultured for 5 days in media alone. Cell turnover and survival were measured by flow cytometry (Figure 24). In the absence of stimulation, the majority of peritoneal cells died after 5 days of culture, with only \~20-30\% of the input cells surviving. Knockout of IL-10 resulted in a significant reduction in the proportion of live cells to \~5-10\% and appeared
to particularly affect the B1a cell population in both B6 and B6.NZBc4m mice, as the proportion of these cells within the live cell population was reduced in the absence of IL-10 (Figure 24). Interestingly, in B6.NZBc4m mice, neither the expansion of CD5$^+$ B cells nor their enhanced turnover capacity as compared to B6 B cells was affected by the IL-10 knockout (Figure 24). These findings suggest that IL-10 is required for peritoneal CD5$^+$ B cell survival but has no effect on the proliferative capacity of these cells.
Figure 24: IL-10 is required for cell survival but has no impact on the proliferation of peritoneal B1a cells.

Peritoneal B cells were harvested and stained with CFSE as described in the Materials and Methods. Cells were cultured without stimulation for 5 days and homeostatic turnover measured by flow cytometry. (A) Representative gating of live (PI, Doublet-excluded), B1a (B220<sup>lo</sup>CD19<sup>+</sup>CD5<sup>+</sup>) and proliferating (CFSE<sup>lo</sup>) cells is shown. Frequency of all live cells (B) and proportion of B1a cells within the live population (C) were measured by flow cytometry as a proportion of total events. The frequency of B1a cells (D) and proliferated B1a cells (E) as a proportion of live cells was measured by flow cytometry. Each point represents a single mouse, with the lines for each group representing the median. Statistical analyses were carried out using a Mann-Whitney U test between homozygous and IL-10 knockout animals of the same genetic background. * P < 0.05, ** P < 0.01.
Discussion

In this study, we have further dissected the immunogenetic basis for the expansion of innate-like lymphocytes and suppression of autoimmunity associated with the NZB c4 32 to 151 Mb interval. We show that the genetic locus leading to expansion of CD5⁺ B cell and NKT cell populations is localized to the mid 91-123 Mb region, whereas the predominant locus leading to suppression of autoimmunity is localized to the 123-151 Mb telomeric region. We also identify a critical role for IL-10 in restraining autoantibody production and supporting the expansion of innate-like lymphocytes.

Expansion of splenic and peritoneal CD5⁺ B cells is a well-documented feature of B6.Sle2 congenic mice, which have an introgressed NZM2410 chromosome 4 interval [118,307]. The Sle2 interval has a mixture of homozygous NZB- and NZW-derived genetic material. In contrast to our mice, which have entirely homozygous NZB regions, Sle2 mice have an NZW interval that extends from 55 to 100 Mb and an NZB interval that extends from 100 to 128 Mb. Previous studies examining Sle2 congenic mice have shown that the expansion of B1a and splenic CD5⁺ B cells localizes to the NZB interval and is likely the result of a genetic polymorphism leading to reduced levels of the Cdkn2c inhibitor, p18 [62,294,306]. The NZBe4m region, where we have localized the gene leading to expansion of CD5⁺ B cells, overlaps with this interval and contains within it the p18 gene, providing support for these findings. However, we continue to document a previously unreported expansion of NK and NKT cells in B6.NZBc4 mice. This expansion is likely the result of another unidentified gene within the middle 91 to 123 Mb
NZB-derived region, unique to our mice. Of note, Jak1, which lies downstream of IL-15, IL-22, and IL-7 signaling, is located at 101 Mb on chromosome 4 at the border of the Sle2 NZB interval, and if functionally altered could result in increased homeostatic expansion of innate-like lymphocytes by promoting cytokine signaling [308,309].

Our previous work has shown that the expansion of IL-10-producing B cells correlates with disease suppression in bicongenic B6.NZBc1c4 mice and that transfer of B6.NZBc4 splenic B cells reduces the frequency of pro-inflammatory T cells in B6.NZBc1 lupus-prone recipient mice [301]. Additional adoptive transfer experiments provided support for a potential role of CD5+ B cells in this suppression [301]. Therefore, we anticipated that creation of bicongenic mice with a shorter telomeric chromosome 4 interval spanning 123 to 151 Mb, which lacked expansion of these cells, would result in little or no suppression. To our surprise, B6.NZBc1c4t maintained suppression of anti-ssDNA and anti-dsDNA antibodies, despite an absence of CD5+ B cell expansion. However, these mice did exhibit an increase in anti-chromatin IgG antibodies, as compared to mice with the longer NZB c4 congenic interval, suggesting that expansion of the CD5+ B cell compartment may lead to somewhat enhanced suppression. While these data indicate that expansion of CD5+ B cells is not required for suppression, they do not rule out a role for the CD5+ B cell population in this process, given our previous adoptive transfer results. It is possible that the CD5+ B cell population in B6.NZBc4t has altered function leading to enhanced suppressive activity. Along these lines, previous studies have shown that B6.Sle2 mice have impaired receptor editing of nuclear antigen-reactive
B cells [64,87]. It is possible that this leads to increased numbers of B cells with low affinity for nuclear antigens that are preferentially selected into the CD5⁺ regulatory B cell compartment. Alternatively, CD5⁺ B regulatory cells may not be the sole mechanism mediating suppression of autoimmunity in B6.NZBc1c4 bicongenic mice, as a genetic locus that leads to decreased autoantibody production in a graft vs host autoimmunity model has been identified in the NZB-derived Sle2 region located between 115 to 128Mb [310,311]. This suppression has been shown to be mediated by non-lymphoid bone marrow-derived populations and may result from altered function of the G-CSFR.

In support of our hypothesis that CD5⁺ IL-10-producing B cells are important to disease suppression, knockout of IL-10 in B6.NZBc4 mice resulted in a breach of tolerance with enhanced production of IgM autoantibodies and an increase in MZ B cells. It is possible that the increase in MZ B cells could promote autoantibody production and autoimmunity in these mice, as shown by others [312–314]. It is also tempting to speculate that the production of autoantibodies in IL-10 knockout B6.NZBc4 mice arises from the presence of the previously identified Sle2 receptor editing defect. In this context, loss of IL-10 in low affinity nuclear antigen-reactive B cells could convert these cells from a regulatory to a stimulatory phenotype. Alternatively, IL-10 may play a role in lineage commitment, with cells that are normally selected into the CD5⁺ B cell compartment being selected into the marginal zone compartment in the absence of IL-10, where they could then become activated to produce autoantibodies. As a caveat, it is important to remember that the ablation of IL-10 was a global knockout that could alter
the function of numerous cells types including IL-10 producing Tregs, Tfregs, iNKT10 and others that could contribute to the overall phenotype. Although we believe from our previous work that CD5⁺ B cells are the most pertinent population in our model of disease, we have not ruled out the role of these other IL-10 dependent suppressive populations.

Surprisingly, loss of IL-10 also resulted in a number of unanticipated effects on the survival and/or selection of innate-like lymphocytes. In our congenic mice, knockout of IL-10 ablated the expansion of splenic and peritoneal CD5⁺ B cells as well as NKT cells. Previous work examining the impact of an IL-10 knockout on NZB mice showed no changes in peritoneal B1a cell numbers but greatly reduced development of “malignant” CD5⁺ B1 cells in the blood and spleen [315]. In another study, administration of anti-sense IL-10 oligonucleotide induced apoptosis and cell cycle disruption in malignant B1 clones from NZB mice, while increasing cyclin E, D2, and A, and reducing p27 levels [316]. Our data provides support for the role of IL-10 as a survival factor for CD5⁺ B cells, but suggests that for non-malignant CD5⁺ B cells, IL-10 does not appear to be required for cellular proliferation. Indeed, the enhanced turnover capacity of peritoneal B1a cells from B6.NZBc4m mice was retained despite the absence of IL-10. Although we have not directly examined the impact of IL-10 knockout on the survival of other innate-like lymphoid populations, it is likely that this cytokine plays a similar role for these cells, as the proportion of NK and NKT cells was also markedly reduced in the absence of IL-10.
In summary, we have localized the expansion of CD5^+ B and NKT cells to the NZBe4m interval, identified a critical role for IL-10 in supporting this expansion, and in its absence, unmasked a minor breach in tolerance in B6.NZBe4 mice. Although the effect of these losses on autoimmunity was modest in B6.NZBe4 mice, other groups have shown that the gain or loss of IL-10 competent regulatory B or NKT cells can have profound effects on disease [186,235,280,282,285,290,302,317–319]. Our data suggests that IL-10 may act through multiple mechanisms to prevent the progression of autoimmunity, and our work adds to this literature by highlighting the novel role it may play in supporting the expansions of suppressive populations.
CHAPTER IV

iNKT cell activation is critically dependent on homotypic trans-Ly108 interactions

Yuriy Baglaenko\textsuperscript{1,2}, Mayra Cruz Tleugabulova\textsuperscript{2}, Eric Gracey\textsuperscript{1,2}, Nafiseh Talaei\textsuperscript{1,2}, Kieran Patricia Manion\textsuperscript{1,2}, Nan-Hua Chang\textsuperscript{1}, Thierry Mallevaey\textsuperscript{2#} & Joan E. Wither\textsuperscript{1,2,3}

\textsuperscript{1} Krembil Research Institute, University Health Network, Toronto, Ontario, Canada
\textsuperscript{2} Department of Immunology, University of Toronto, Toronto, Ontario, Canada
\textsuperscript{3} Department of Medicine, University of Toronto, Toronto, Ontario, Canada

(All experiments were performed by Y. Baglaenko; Erg2 and PLZF expression was assessed by M. Cruz Tleugabulova; all other authors assisted with experiments or helped with experimental design and analysis)
Abstract

Invariant natural killer T cells (iNKT) are innate lymphocytes that respond to glycolipids presented by the MHC-Ib molecule CD1d and are rapidly activated to produce large quantities of cytokines and chemokines. iNKT cell development uniquely depends on interactions between double positive thymocytes that provide key homotypic interactions between signaling lymphocytic activation molecules (SLAM) family members. However, the role of SLAM receptors in the differentiation of iNKT cell effector subsets and activation has not been explored. Here, we show that C57BL/6 mice containing the New Zealand Black Slam locus have profound alterations of Ly108, CD150 and Ly9 expression, associated with iNKT cell hypo-responsiveness. This loss-of-function was only apparent when both dendritic cells (DCs) and iNKT cells had a loss of SLAM receptor expression. Using small interacting RNA knockdowns and peptide blocking strategies, we demonstrated that trans-Ly108 interactions between DCs and iNKT cells are critical for robust activation. Ly108 co-stimulation similarly increased human iNKT cell activation. Thus, in addition to its established role in iNKT cell ontogeny, Ly108 regulates iNKT cell function in mice and humans.
Introduction

Natural Killer T (NKT) cells are innate lymphocytes that recognize glycolipids presented by the major histocompatibility class Ia molecule CD1d [203,205]. Among these, type I or invariant (i) NKT cells, express semi-invariant T cell receptors (TCR) formed by a canonical Vα14-Jα18 (Vα24-Jα18 in humans) TCRα chain paired with Vβ8s, Vβ7, or Vβ2 (Vβ11 in humans). iNKT cells respond within minutes following antigen recognition to produce large quantities of cytokines including IFN-γ, IL-4, TNF-α, IL-17A, and IL-10, that influence the ensuing immune response [204]. Unlike conventional T cells, which are selected from double positive (DP) thymocytes by peptide-MHC complexes expressed by thymic epithelial cells, iNKT cells develop through DP-DP thymocyte interactions, where the presentation of unidentified self lipids by CD1d to the TCR plays an essential role [203,320].

iNKT cells acquire a memory/activated phenotype in the thymus and then seed lymphoid and non-lymphoid tissues such as the spleen, lymph nodes, liver, lungs, and skin. Recent work has shown that iNKT cells, similar to T helper cells, can be subdivided into three distinct lineages, NKT1, NKT2, and NKT17, and discriminated by expression of cell surface markers or transcription factors [214,215]. Expansion and preferential activation of these subsets can drive differences in the immune response [212,213]. Their phenotype and anatomical location afford iNKT cells with potent adjuvant and regulatory functions that influence the outcome of the immune response in a number of diseases including bacterial infections, cancer and autoimmunity. iNKT cells exert protective
functions in mouse models of systemic lupus erythematosus (SLE). In studies of NZB/W F1 and MRL/lpr animals, administration of α-galactosylceramide (αGalCer), a prototypical iNKT cell antigen, attenuated disease progression [321,322]. Similarly, genetic ablation of CD1d, which is critical for iNKT cell development, exacerbated disease on the NZB/W F1 genetic background [230]. In humans, a reduction of peripheral iNKT cell frequency and altered function have been reported in SLE patients, although it is not clear whether these changes participate in disease development, or are a consequence of the strong inflammatory response [235,238]. However studies of first-degree relatives in SLE patients suggest that iNKT cell dysfunction precedes autoimmunity suggestive of a causative role in disease [238]. Similarly, various solid state and hematological cancer patients have been shown to have reductions in iNKT cell numbers and function which inversely correlated with prognosis [323–329]. Although iNKT cells are protective in these diseases, increased iNKT cell frequencies and activation have been correlated with disease severity in asthma patients, suggesting a pathogenic role [330]. Understanding the factors that control iNKT cell development and function are therefore critical to devising immunotherapies targeting these cells in a multitude of disorders.

In addition to TCR-mediated signals, homotypic interactions of members of the signaling lymphocyte activation molecule (SLAM) receptor family, provided through DP-DP interactions, are critical for iNKT cell development [219,331]. The SLAM family comprises 9 related immunoglobulin-like domain-containing proteins implicated in B, T,
and NK cells responses. With the exception of CD84 and CD244, SLAM proteins are activated through homotypic trans or cis interactions and signal through downstream adaptors such as SAP, SHP-1/2, SHIP-1, ERT-2, and EAT-2 [239]. Work has shown that the expression and signaling of these molecules is especially critical to the development of innate T cells, specifically iNKT cells. Using congenic and knockout mice, various groups have identified key roles for CD150 (Slamf1), Ly108 (Slamf6), and Ly9 (Slamf3) in controlling iNKT cell development [203,219,220,244,262]. The loss of these molecules at the DP stage impedes iNKT cell development leading to reduced peripheral frequencies. For iNKT cells, signaling through Ly108 and CD150 has been shown to promote a SAP/Fyn-dependent signaling cascade that results in the upregulation of the transcription factors, Egr2 and PLZF, which are critical to iNKT lineage commitment [219,221–223,332]. It is likely that the SLAM molecules play a similar role in humans, since patients with mutations in the adaptor SAP, have a significant loss of iNKT cells [218]. Although there is clear support for the role of Ly108 and CD150 in iNKT cell development, the exact role of SLAM molecules in iNKT cell effector functions has not been addressed.

The autoimmune prone New Zealand Black (NZB) and derivative congenic strains of mice have been used extensively to study the genetics of SLE, identifying several critical genes involved in pathogenesis. One of the genetic regions tightly linked to SLE contains the Slam/Cd2 locus [241]. Studies have shown that various inbred strains of mice have different expression of SLAM family members and related isoforms [241].
Of note, studies with congenic B6.Sle1b mice, derived from the New Zealand Mixed (NZM) strain, have previously identified three differentially expressed Ly108 isoforms (Ly108-1, Ly108-2, and Ly108-H1) with different signaling capabilities [240,263,269,333]. How these isoforms impact iNKT cell development and function is still unknown.

In this study, we investigated the role of Ly108 on iNKT cell activation in both mice and humans. We found that C57BL/6 mice containing the NZB Slam locus have profound alterations of Ly108, CD150 and Ly9 expression, associated with iNKT cell hypo-responsiveness upon antigenic stimulation. This functional defect was only apparent when both dendritic cells (DCs) and iNKT cells expressed NZB SLAM molecules. Using small interacting RNA knockdowns and peptide blocking strategies, we demonstrated that trans-Ly108 interactions between DCs and iNKT cells are critical for optimum activation. Finally, similar to our findings in mice, Ly108 co-stimulation potentiated human iNKT cell activation and cytokine secretion. Thus, Ly108 regulates iNKT cell function in mice and humans.
Materials and Methods

**Ethics Statement.** Mice were housed in a Canadian Council on Animal Care approved facility at the Krembil Research Institute in the Krembil Discovery Tower, part of the University Health Network. All experiments performed in this study were approved by the Animal Care Committee of the University Health Network (Animal Use Protocol 123).

**Mice.** Wildtype C57BL/6 (B6) and NZB mice were purchased from Jackson Laboratory and Harlan Sprague Dawley (Blackthorn, England), respectively. Congenic mice were generated as previously described [334]. B6.NZB(171-177) mice are identical to previously referenced C1(96-100) or B6.NZBc1(171-177) animals. Only female mice were used for all experiments in this study.

**Cell Lines.** The derivation of the NKT cell hybridoma, DN32.D3, has been previously reported [335]. Ly108+ and Ly108- NKT cell hybridomas were generated as follows: The TCRαβ-/- 5KC-78.3.20 hybridoma was used to generate a CD4- and CD1dlow 6KC hybridoma. 6KC hybridomas were then transduced with the retroviral plasmids MIGR1 and pDSRed containing the iNKT TCR Va14i-DQβ [336] and the Ly108-1 gene, respectively, by spinfection at 4500 x g, 37°C for 90 minutes. Transduced cells were sorted for similar expression of TCRβ and the presence or absence of Ly108 expression on the FACSARia (BD). Cell lines were cultured in complete RPMI 1640 (RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS (Wisent), L-glutamine (Gibco), non-essential amino acids (Gibco), and penicillin and streptomycin (Gibco)).
**Flow Cytometry.** Splenocytes were harvested and RBC lysed, as previously described [74]. Briefly, a million cells were incubated with 1 μg/ml mouse IgG (Sigma-Aldrich) for 15 minutes on ice prior to staining with directly conjugated or biotinylated mAbs for 30 minutes on ice. The following antibodies were used for primary staining (all purchased from BioLegend, BD Biosciences, or Ebiosciences): FITC-conjugated anti-TCRβ(H57-597), -CD3ε(145-2C11), -CD150(TC15-12F12.2), and -CD11b(M1/70); AlexaFlour 488-conjugated anti-CD150(TC15-12F12.2), and -TCRβ(H57-597); PE-conjugated anti-NK1.1(PK136), -Ly108(330-AJ), -B220(RA3-6B2), -CD1d(1B1); -Ly9(Ly9ab3), and -CD44(IM7); PE-Cy7 anti-CD19(6D5), -CD8(53-6.7), and -CD44(IM7); APC-conjugated anti-CD150(TC15-12F12.2); APC-Cy7 conjugated anti-CD11b(M1/70); BV605 conjugated anti-B220(RA3-6B2), -CD3ε(145-2C11), and -CD11c(N418); Pacific Blue-conjugated anti-CD4(GK1.5), -B220(RA3-6B2), and -MHCII(M5/114.15.2); and biotinylated anti-Ly49C/I(5E6). Cells were washed twice and biotinylated samples stained with SA-Pacific Blue (Life Technologies). Dead cells were identified by staining with 0.6 μg/mL Propidium Iodide (Sigma Aldrich). APC-conjugated or PE-conjugated unloaded and PBS-57-loaded mouse CD1d tetramers (Tet) were generously provided by the National Institutes of Health Tetramer Core Facility. Events were collected on a BD LSRII or BD FACSCanto and analyzed using FlowJo software (Tree Star Inc).

**Intracellular Flow Cytometry and Transcription Factor Staining.** In experiments examining αGalCer or BMDC injected animals, splenocytes were harvested and stained as described above. Cells were then fixed with BD Cytofix/Cytoperm for 30 minutes on ice and subsequently stained for 30 minutes with PE-conjugated anti-IL-4 (11B11) or
anti-IFNγ (XMG1.2) in BD Permwash. Cells were washed twice and events collected on
a BD LSRII or BD FACSCanto and analyzed using FlowJo software (Tree Star Inc). For
staining of transcription factors, 10^6 splencotyes or thymocytes were stained for
extracellular markers as described above and subsequently fixed with
Foxp3/Transcription Factor Fixation/Permeabilization Buffer. Cells were washed and
stained with APC conjugated anti-SAP, PerCPe710 conjugated anti- RORγT (B2D), PE
coujugated anti-PLZF(Mags.21F7), and PECy7 conjugated anti-GATA3 (TWAJ). Events
were collected on a BD LSRII or BD FACSCanto and analyzed using FlowJo software
(Tree Star Inc)

**Activation of NKT cells in vivo.** Mice were tail vein injected with 2 μg of αGalCer
resuspended in PBS or an equal volume of DMSO/PBS and sacrificed 90 minutes later.
Splencocytes were immediately harvested, as described earlier, and stained with Infrared
Live/Dead (Life Technologies) for 30 minutes. Splenocytes were then stained for
extracellular markers and intracellular cytokines as described earlier.

**Bone-Marrow Derived Dendritic Cell (BMDC) Generation.** Bone marrow cells were
isolated by flushing the femurs of 6–10-wk-old B6 or B6.NZB(171-177) mice. RBC were
lysed and cells resuspended at 1-2 x 10^6 cells/ml in complete RPMI and cultured in 6 well
plates (Corning) for 9 days with 200 ng/ml recombinant human FLT3L (Biolegend) to
expand BMDCs. Non-adherent cells, harvested at day 9, were used in all subsequent
experiments.

**Activation of NKT cells with BMDCs in vivo.** Cultured BMDCs were plated in a 24
well plates in 10% complete RPMI and pulsed overnight with 100 ng/mL αGalCer
(Avanti Polar Lipids) or DMSO/PBS control. Following pulsing with glycolipid, BMDCs were recovered and washed extensively in PBS. 1 million BMDCs were injected into the tail vein of recipient animals. Mice were sacrificed 6 hours after injection and splenocytes immediately isolated and stained for intracellular cytokine production as described above.

Transfection of BMDCs with siRNA and Ly108Fc Staining. 5 × 10⁶ rhFLT3L expanded BMDCs were electroporated with siGENOME Non-Targeting Control small interfering RNA (siRNA; scrambled control) or siGENOME SMARTpool Slamf6(Ly108) siRNA (GE Healthcare Dharmacon) at a final concentration of 4 μM. After transfection, cells were incubated in 24 well plates (BD Biosciences) in complete RPMI supplemented with 20% FCS for 24 h. BMDC were washed collected, washed, and re-plated in 24 well plates for subsequent experiments. For blocking trans-Ly108 interactions, rhFLT3L expanded BMDCs were incubated with Ly108Fc (R&D Systems) [333] or control IgG2aFc (R&D Systems) at a concentration of 10 μg/mL in PBS. Cells were washed twice with PBS then tail vein injected into mice.

DC-NKT cell conjugations and IL-2 co-cultures. BMDCs were generated and pulsed overnight with 100 ng/mL, as described above. αGalCer or control pulsed BMDCs were stained with PKH26 (Sigma) following the manufacturers protocol. Briefly, cells were washed with PBS, stained with membrane dye for 5 minutes at 37 degrees, and quenched with complete RPMI. Simultaneously, Ly108- or Ly108+NKT cell hybridomas were identically stained with PKH67 (Sigma). Following washes, 2.5 x 10⁶ BMDCs were mixed with 2.5 x 10⁶ hybridoma cells in 5ml culture tubes in complete RPMI 1640. Cells were spun down for 5 minutes at 300g and incubated at 37 degrees for 30 minutes. Cells
were immediately fixed with an equal volume of BD Cytofix/Perm for 30 minutes on ice and then washed once before events were collected by flow cytometry. For IL-2 production, 10^4 BMDCs were similarly generated, pulsed with 10 ng/mL, 30 ng/mL or 100 ng/ml of αGalCer and cultured overnight with 10^5 DN32.D3 or Ly108- or Ly108+ NKT cell hyrbidomas in 96 well flat bottom plates. After culture, plates were spun down and supernatants collected. IL-2 was measured using an IL-2 ELISA kit (Biolegend).

**Quantitative Real-time PCR.** RNA was purified from rhFLT3 cultured BMDCs, total thymocytes, or magnetically sorted naïve T cells (Miltenyi) using a Total RNA Mini Kit (FroggaBio). RNA was converted to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Sigma). Quantitative real-time PCR was performed with SYBR Green Master Mix on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using default cycling conditions. Primer sequences were as follows: GAPDH 5’-TGCACCACCAACTGCTTAG-3’ and 3’-GGATGCAGGGATGATGTTC-5’; Ly108-1 5’-CAGGGAACACTGTGTATGCACA-3’ and 3’-TTCGGCCCTCTCTGGAATGAT-5’; Ly108-2 5’-CCAGAGAGAAGAAGTGCTTT-3’ and 3’-CTCAAGTCAGTGCTCTTTC-5’; and Ly108-H1 5’-CAGGGAACACTGTGTATGCACA-3’ and 3’-AGCATTGTATTTAATTCTCAAGTACTC-5’. Gene expression was analyzed using the ∆∆CT method and normalized to either β-actin or GAPDH expression.

**EGR2 and PLZF stimulation.** Upregulation of Egr2 and PLZF on pre-selection DP thymocytes was measured as previously described [333]. Briefly, 96 well plates were coated with 1 μg/ml anti-CD3 (2C11) + 5 μg/ml anti-IgG2a Isotype (eBM2a) or 1 μg/ml
anti-CD3 (2C11) + 5 μg/ml Ly108 (13G3-19D) overnight at 37°C. Thymocytes from B6 and B6.NZB(171-177) were incubated with biotinylated antibodies CD3, CD25 and CD44 for 15 minutes at 23°C and DP thymocytes were enriched using streptavidin magnetic beads (Miltenyi). 0.5 million cells were then plated per well and collected at 2 hours to measure Egr2 expression or at 48 hours to measure PLZF expression. At each time point, cells were stained with surface markers CD4 and CD8, fixed using FoxP3 fixation/permeabilization kit (eBiosciences), stained intracellularly with Egr2 or PLZF, and analyzed by flow cytometry on a LSR Fortessa (BD).

Isolation and Stimulation of Peripheral Blood Mononuclear Cells (PBMC). Male healthy controls (n = 7) were recruited between the ages of 18 and 35 years with no family history of autoimmune disease. The study was approved by the Research Ethics Board of the University Health Network and all subjects provided informed consent. PBMCs were isolated over a Ficoll (GE Healthcare) gradient, lysed to remove residual RBCs, and washed once at room temperature. Cells were re-suspended in complete RPMI 1640 and 2 million cells per well rested overnight in 96 well U-bottom plates (BD Biosciences). Cells were washed once and stimulated with 100 ng/ml αGalCer (Avanti Polar Lipids) in the presence of 0.2 μg/ml GolgiPlug (BD Biosciences). Control IgG1 (MOPC-21) and anti-Ly108 (NT-7) were simultaneously added to the cultures at varying concentration. PBMCs were stimulated overnight, washed twice, placed in 96 well V-bottom plates and stained for extracellular cell surface markers.

Flow Cytometry on PBMCs. For measurement of cell surface Ly108 expression, 2 million freshly isolated PMBCs were plated in 96 well V-bottom plates and stained with
anti-NTB-A (NT-7) for 30 minutes on ice followed by APC conjugated anti-mouse IgG1 (RMG1-1) for 30 minutes on ice. Cells were subsequently stained with the following directly conjugated antibodies: FITC conjugated anti-CD4(OKT4); PE conjugated human CD1d tetramer (NIH); PE-Cy7 conjugated anti-CD45RA(HI100); PB conjugated anti-CD19(HIB19); BV605 conjugated anti-CD3(UCHT1); and APC-Cy7 conjugated anti-CD8(OKT8). Dead cells were identified by staining with 0.6 μg/mL Propidium Iodide (Sigma Aldrich). A minimum of 1 million events were collected on a BD FACSCanto and analyzed using FlowJo software (Tree Star Inc). For detection of intracellular cytokines, rested and stimulated PBMCs were transferred into V-bottom plates and stained with NIR Live/Dead stain for 30 minutes on ice. Cells were then stained with the following directly conjugated antibodies for 30 minutes on ice: FITC conjugated anti-CD3(UCHT1); PE conjugated human CD1d tetramer (NIH); and PerCP conjugated anti-CD19(HIB19). Cells were then fixed with BD Cytofix/perm and stained with BV605 conjugated anti-TNFα(Mab11) and APC conjugated anti-IFNγ(4S.B3) in BD permwash. A minimum of 1 million events were collected on a BD FACSCanto and analyzed using FlowJo software (Tree Star Inc).

**Statistics.** For comparisons of differences between three or more groups, a Kruskal-Wallis test was used followed by Dunns’ post-test for multiple comparisons. For comparison between two groups Mann-Whitney U tests were performed. For analysis of paired responses, non-parametric Friedman tests were performed. All statistical analyses were performed using GraphPad software (La Jolla, CA, USA).
Results

A Slam-containing NZB 171-177 Mb chromosome 1 interval alters iNKT cell homeostasis

We previously reported that introduction of an NZB chromosome 1 interval spanning 133 to 177 Mb onto the C57BL/6 (B6) background caused a reduction of splenic iNKT cell frequency [74]. However, these mice develop severe autoimmunity, confounding interpretation of these results. To circumvent this, we analyzed subcongenic mice containing a shorter (171-177) chromosome 1 interval containing the genetic locus encoding the SLAM family of molecules (Figure 25). We have previously reported that these mice do not develop overt autoimmunity but do have a slight breach of tolerance to nuclear antigens [334]. Similar to B6.NZB(133-177) mice, B6.NZB(171-177) mice have reduced splenic iNKT cell frequencies. However, due to a previously reported increase in the number of splenocytes in this strain, the total numbers of splenic iNKT cells were not different from B6 mice (Figure 25). Using expression of CD4 and NK1.1, we assessed the frequency of peripheral iNKT cell subsets. There was a clear shift in the frequency of subsets in B6.NZB(171-177) mice with a decrease in NK1.1$^+$ iNKT cells and an increase in NK1.1$^-$ iNKT cells indicating a possible increase in NKT2s and decrease in NKT1s (Figure 25). Interestingly, expression of CD44 was significantly reduced suggestive of a possible defect in PLZF signaling (Figure 25) [337]. Ly49C/I, a receptor associated with chronic antigenic stimulation [338], was also slightly lower on iNKT cells from B6.NZB(171-177) mice (Figure 25). These data suggest that peripheral iNKT cell subsets from B6.NZB(171-177) mice are altered and may have an overall decrease in activation.
Figure 25: Reduced splenic iNKT cell frequency and skewed peripheral subsets in B6.NZB(171-177) mice.

(A) Schematic representation of introgressed NZB chromosome 1 intervals in B6.NZB(133-177) and B6.NZB(171-177) mice. (B) The frequency (as a proportion of CD19- cells) and number of splenic iNKT cells (TCRβ+$\text{Tetramer}^+$), as measured by flow cytometry. (C) Representative gating of peripheral NKT cell subsets from 21 week old B6 mice based on expression of CD4 and NK1.1. (D) Summary of peripheral iNKT cell subsets as a frequency of splenic iNKT cells. (E&F) Geometric MFI of CD44 on iNKT cells was measured by flow cytometry and normalized to B6 levels. (G) Frequency of Ly49C/I$^+$ iNKT cells. Each data point represents an individual mouse with bars representing population medians. Mice in B were aged to 4 - 5 months. Mice in D-G are 6-8 weeks of age. All data was analyzed by non-parametric Mann-Whitney U test. * p < 0.05, ** p < 0.01, *** p < 0.001.
Given the differences in peripheral iNKT cell subsets and the previously published role of SLAM molecules in iNKT cell development, we investigated whether iNKT cell development was altered in the thymus of B6.NZB(171-177) mice. Similar to the spleen, thymic iNKT cell frequencies were reduced, whereas total numbers remained unaffected (Figure 26). Of note, the relative and absolute numbers of double positive (DP) thymocytes were increased in B6.NZB(171-177) compared to B6 mice (Figure 26). Surprisingly, CD1d expression on B6.NZB(171-177) thymocytes was reduced to approximately half compared to B6 thymocytes (Figure 26). This expression level is similar to what is found in CD1d heterozygous mice, which have been previously shown to have no impact on peripheral iNKT cell numbers, indicating that this change by itself is likely insufficient to result in the reduced proportions of iNKT cells in B6.NZB(171-177) mice[339,340]. Consistent with previous reports suggesting that the Slam locus is polymorphic in NZB mice, expression of CD150 (Slamf1) and Ly108 (Slamf6) on DPs, which have been previously reported to play an important in iNKT cell selection and/or expansion [219], were significantly lower on B6.NZB(171-177), as compared to B6 (Figure 26). In contrast, Ly9 (Slamf3) expression, which may restrict iNKT2 differentiation [220], was increased in these mice. The levels of SAP, the central SLAM signaling adaptor essential for iNKT cell development, were similar on DP thymocytes of both strains (Figure 26). Taken together, these findings argue that the reduced frequency of iNKT cells in B6.NZB(171-177) mice is most likely the result of altered SLAM expression.
Figure 26: Reduced thymic iNKT cell frequencies and alteration in Ly108, CD150, and Ly9 expression in B6.NZB(171-177) mice.

(A & B) The frequency and number of thymic NKT cells (TCRβ^+Tet^+) and double positive(CD4^+CD8^+) thymocytes. (C) Geometric mean fluorescence intensity of CD1d on thymocytes as measured by flow cytometry and expressed relative to B6 levels (n ≥ 3 per experiment). (D) The geometric mean fluorescence intensity of Ly108, CD150, and Ly9 was measured by flow cytometry on double positive thymocytes and expressed relative to average B6 levels in each experiment (n ≥ 3 per experiment). (E) Expression of SAP in double positive thymocytes from B6(blue) and B6.NZB(171-177)(red) mice. Light gray represents the FMO control. All mice were age 6-8 weeks. Each data point represents an individual mouse with bars representing population medians. Data shown are pooled from 3 independent experiments. All data was analyzed by Mann-Whitney U tests comparing B6 and B6.NZB(171-177) groups. * p < 0.05, ** p < 0.01, *** p < 0.001.
The NZB locus on chromosome 1 controls iNKT cell effector differentiation

To further explore the impact of the polymorphic Slam-containing NZB chromosome 1 locus on iNKT cell effector differentiation, we took advantage of the well-characterized transcription factors PLZF and RORγt to determine the frequency of iNKT1 (PLZF\textsuperscript{lo} RORγt\textsuperscript{neg}), iNKT2 (PLZF\textsuperscript{hi} RORγt\textsuperscript{neg}), and iNKT17 (PLZF\textsuperscript{int} RORγt\textsuperscript{pos}) subsets in the thymus and spleen. In both tissues, B6.NZB(171-177) mice had an increase in iNKT2 cells and a reduction of iNKT1 cells compared to B6 mice, whereas iNKT17 cells were unaffected (Figure 27). The higher frequency of iNKT2 cells was confirmed by higher expression of the signature transcription factor GATA3 in B6.NZB(171-177) iNKT cells. We also assessed expression of the traditional cell surface markers CD24, CD44 and NK1.1 in the thymus (Figure 27). We found a reduced frequency of NK1.1\textsuperscript{+} iNKT cells (reminiscent of iNKT1 cells) and increased frequency of CD24\textsuperscript{+} iNKT cells (Figure 27). Finally, reduction of CD1d expression and increase in iNKT2 cells has previously been linked with increased Vβ7 usage in studies of CD1d knockout heterozygous mice, suggestive of a selective pressure for high avidity TCRs [341]. In line with the observed loss of CD1d expression and skewing in effector differentiation, we found that B6.NZB(171-177) iNKT cells had an increase in Vβ7 gene segment usage compared to B6 iNKT cells (Figure 27). These data suggest that the NZB Slam locus favors the development of iNKT2, while limiting development of iNKT1 cells in the thymus and that these changes are preserved in the periphery. This concept is further supported by the observation that the proportion of splenic NK1.1\textsuperscript{+} iNKTs is decreased whereas that of
Figure 27: B6.NZB(171-177) mice have skewed inKt cell development and altered
NKT cells subsets.

(A-C) Representative gating and frequencies of NKT1(RORγT+PLZF<sub>low</sub>), NKT2(RORγT−
PLZF<sub>high</sub>PLZF<sup>+</sup>GATA3+), and NKT17(RORγT+PLZF<sup>+</sup>) subsets within the thymic and
splenic NKT cell population (Tet<sup>+</sup>TCRβ<sup>+</sup>). (D) Representative gating of the different
stages of NKT development in the thymus as based on CD24, NK1.1, and CD44
expression. (E) Frequency of NKT cells in each stage of development expressed as a
proportion of total thymic Tet<sup>+</sup> cells. (F) Vβ7 and Vβ8 usage in thymic iNKT cells. All
mice were age 6-8 weeks. Each data point represents an individual mouse with bars
representing population medians. Data shown are pooled from at least 3 independent
experiments. All data was analyzed by Mann-Whitney U tests comparing B6 and
B6.NZB(171-177) groups. * p < 0.05, ** p < 0.01, *** p < 0.001.
NK1.1− iNKTs is increased (Figure 25), which are thought to roughly correlate with iNKT1 and iNKT2 cell subsets, respectively.

*Altered SLAM receptor expression is associated with defective peripheral iNKT activation in B6.NZB(171-177) mice*

We next assessed whether the NZB(171-177) locus is associated with altered iNKT cell function. Mice were given αGalCer intravenously and production of IFN-γ and IL-4 by splenic iNKT cells assessed 90 min later. There were markedly reduced proportions of IFN-γ and IL-4-producing iNKT cells in B6.NZB(171-177) as compared to B6 mice (Figure 28). These changes occurred despite the increased frequency of GATA3+ iNKT2s in B6.NZB(171-177) mice, described above. In addition, the decreased proportion of IFN-γ producing cells was more profound than would be expected for the approximate 10% reduction in iNKT1 cells in these mice. Surprisingly, B6 and B6.NZB(171-177) splenic iNKT cells produced identical amounts of IFN-γ and IL-4 following stimulation with the phorbol ester PMA and Ca2+ ionophore Ionomycin, which bypass TCR stimulation (Figure 28). This result suggested that Slam family molecules might influence TCR-mediated activation of iNKT cells. Of note, this defective cytokine production was not a result of altered levels of CD1d expression on dendritic cells (DCs) (Figure 28). However, as in the thymus, SLAM receptor expression was greatly altered. B6.NZB(171-177) splenic iNKT cells and DCs had lower Ly108 expression, whereas the levels of Ly9 and CD150 were normal or increased in these cells subsets (Figure 28). Once again, SAP
Figure 28: iNKT cells from B6.NZB(171-177) mice are functionally defective and have deficiencies in SLAM family expression.

(A) Representative gating of in vivo stimulated splenic iNKT cells (TCRβ^+Tet^+CD19^). Mice were injected intravenously with 2 μg of αGalCer and sacrificed 90 minutes post injection. Frequency of splenic IFNγ and IL-4 producing iNKT cells as a proportion of NKT cells was measured by intracellular flow cytometry. (B) IFNγ and IL-4 production was measured in splenic NKT cells (TCRβ^+Tet^+CD19^) after three hours of stimulation with PMA and Ionomycin in the presence of GolgiStop. (C) Representative staining of CD1d and gMFI intensity on MHCII^CD11c^ cells relative to B6 levels as measured by flow cytometry. (D) Representative staining of Ly108, CD150, and Ly9 on splenic NKT cells (TCRβ^Tetramer^CD19^) and dendritic cells (MHCII^CD11c^). Blue shaded histograms represent B6 mice and red shaded histograms represent B6.NZB(171-177) mice. (E & F) The geometric median intensity of Ly108, CD150, and Ly9 on splenic iNKT cells and DCs. Data is expressed relative to average B6 levels in each experiment (n ≥ 3 per experiment). (G) Relative expression of SAP in B6 and B6.NZB(171-177) splenic iNKT cells. Mice in A were age 4-5 months. Mice in B-G were age 6-8 weeks. Each data point represents an individual mouse with bars representing population medians. Data shown were pooled from 3 independent experiments. All data was analyzed by Mann-Whitney U tests comparing B6 and B6.NZB(171-177) groups. * p < 0.05, ** p < 0.01, *** p < 0.001.
was expressed at similar levels in splenic iNKT cells from both strains (Figure 28). These findings raise the possibility that the B6.NZB(171-177) iNKT cell hypo-responsiveness observed following αGalCer injection results from altered expression of Ly108 on iNKT cells and/or DCs.

*Homotypic Ly108 interactions between DCs and iNKT cells are critical for iNKT cell activation*

To investigate whether abnormalities at the DC-iNKT synapse lead to ineffective iNKT cell activation in B6.NZB(171-177) mice, B6 and B6.NZB(171-177) bone-marrow derived dendritic cells (BMDCs) were pulsed with αGalCer, and injected into B6 or B6.NZB(171-177) recipient mice as outlined in Figure 29. CD69 expression and IFN-γ or IL-4 production by iNKT cells were then assessed 6 hours later using flow cytometry (Figure 29). Surprisingly, both B6 and B6.NZB(171-177) BMDCs equivalently activated iNKT cells in B6 recipients. Likewise, injection of B6 BMDCs could equally stimulate iNKT cells in B6.NZB(171-177) and B6 recipients, suggesting that iNKT cells from B6.NZB(171-177) mice can be effectively activated despite the shift in iNKT cell subsets, provided that they are stimulated by B6 DCs. The impaired activation in B6.NZB(171-177) iNKT cells could only be recapitulated by injection with B6.NZB(171-177) BMDCs, indicating that abnormalities are required on both iNKT and DC populations to lead to a functional defect.
Figure 29: Only injection of B6.NZB(171-177) BMDCs into B6.NZB(171-177) recipients can recapitulate the loss of IFNγ and IL-4 production in iNKT cells.

(A) Schematic representation of BMDC transfer and NKT activation experiments. BMDCs were cultured from B6 (circles) or B6.NZB(171-177) (squares), pulsed overnight with αGalCer and injected into B6 (blue) or B6.NZB (171-177)(red) recipients. Mice were sacrificed 6 hours after injection. (B) Median fluorescence intensity of CD69 on iNKT cells was measured by flow cytometry. Data is expressed relative to average B6 levels. (C,D) Intracellular production of IFNγ and IL-4 was measured as previously described from NKT cells (TCRβ’Tet’). All mice were age 8-10 weeks. Each data point represents an individual mouse with bars representing population medians. All data was pooled from 2 independent experiments and analyzed by non-parametric Kruskal-Wallis with Dunn’s multiple post-tests. * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 30: B6.NZB(171-177) BMDCs have decreased Ly108 expression but no changes in αGalCer presentation or conjugate formation with NKT cells.

(A) Representative gating of rhFLT3L cultured BMDCs separated into the two major populations, B220⁺CD11b⁻ (pDC) and B220⁻CD11b⁺ (mDC). (B-D) Frequencies of CD11c⁺ BMDCs, mDCs, or pDCs were measured by flow cytometry. (E) The geometric mean fluorescence intensity of CD1d was measured by flow cytometry from BMDCs and expressed relative to average B6 values. (F) 10⁴ Cultured BMDCs from B6 (blue) or B6.NZB (171-177)(red) mice were pulsed overnight with 100 ng/mL αGalCer and cultured with 105 DN32.D3 NKT hybridomas. IL-2 was measured from the supernatant following 18 hours of co-culture. Data is pooled from three independent experiments with at least n=3 per group. Bars represent the mean with SEM. (G) Representative staining of Ly108, CD150, and Ly9 on BMDCs from B6 (blue) and B6.NZB(171-177) (red) mice. (H) Geometric mean fluorescence intensity of Ly108, CD150, and Ly9 on BMDCs expressed relative to average B6 values. Data was pooled from three independent experiments with at least three mice per group. Each point represents a single mouse with lines representing population medians. (I) Co-culture of BMDCs stained with PKH26 pulsed with or without αGalCer and Ly108⁻ or Ly108⁺ NKT cell hybridoma cell lines stained with PKH67. (J) The frequency of conjugates was quantified as a ratio of total NKT hybridoma cells by flow cytometry after 30 minutes. Frequency is expressed as BMDC-NKT conjugates/total NKT cells. Filled bars represent αGalCer pulsed BMDCs from B6 in blue and B6.NZB(171-177) in red. Data is pooled from 4 independent experiments with at least n = 3 per condition. Bars represent the mean with SEM. All statistics were carried out using a Mann-Whitney U test between B6 and B6.NZB(171-177) mice. * p< 0.05, ** p < 0.01, ***p <0.05.
Therefore, we compared BMDCs generated from both strains of mice. Generation and differentiation of BMDCs was unaltered between the two mouse strains (Figure 30). To rule out the possibility that αGalCer presentation was altered in B6.NZB(171-177) BMDCs, we measured expression of CD1d (Figure 30). Although expression of this lipid-presenting molecule was slightly reduced in pDCs generated from B6.NZB(171-177) mice, this population represented a minor fraction of the differentiated BMDCs, and expression in mDCs was unchanged. Furthermore, αGalCer-pulsed BMDCs from both strains equally induced IL-2 production by the DN32.D3 iNKT cell hybridoma (Figure 30). In contrast, as before, expression of SLAM receptors was greatly altered with Ly108 consistently reduced in both mDCs and pDCs (Figure 30). Since Ly108 has been implicated in B-T cell conjugate formation, we also tested conjugation efficiency between cultured BMDCs and a Ly108 expressing iNKT cell hybridoma. No change in conjugation efficiency was observed, regardless of Ly108 status or BMDC genotype (Figure 30).

To directly assess whether Ly108 homotypic interactions at the DC-iNKT interface influence iNKT cell activation, we interfered with this pathway through two complimentary approaches. For these experiments we used B6.NZB(171-177) recipient mice as loss of Ly108 only had a functional outcome in these mice (Figure 29). As a first approach, we silenced Ly108 expression in B6 BMDCs using small interfering (si) RNA prior to αGalCer sensitization and injection into B6.NZB(171-177) recipient mice, as
Figure 31: Silencing of Ly108 or inhibition of trans-Ly108 signaling reduces NKT cell activation.

(A) Schematic representation for the experiments carried out in B-E. BMDCs were cultured and Ly108 silenced by electroporation using a pool of siRNA. Cells were rested overnight and then pulsed overnight with αGalCer. BMDCs were injected into B6.NZB(171-177) recipients. Open hexagons represent scrambled control knockdown BMDCs. Half-filled hexagons represent the transfer of Ly108 knockdown BMDCs. Representative knockdown efficiency is shown. (B-D) Median fluorescence intensity of CD69 relative to scrambled controls, IFNγ and IL-4 production was measured by flow cytometry. (E) Schematic representation of the experiments carried out in F-I. As before, BMDCs were pulsed with αGalCer, however, prior to injection into recipient B6.NZB(171-177) cells were incubated with Ly108Fc fusion protein, previously reported in [333], or IgG2aFc control. Open diamonds represent IgG2aFc incubated BMDCs and half-filled diamonds represent Ly108Fc incubated BMDCs. (F-I) As before, median fluorescence intensity of CD69 relative to IgG2aFc controls, IFNγ and IL-4 production was measured by flow cytometry. All mice were age 8-10 weeks. Each data point represents an individual mouse with bars representing population medians. All data was analyzed by Mann-Whitney U tests comparing B6 and B6.NZB(171-177) groups. * p < 0.05, ** p < 0.01, *** p < 0.001.
these DCs were significantly less efficient at activating iNKT cells, as assessed by CD69 depicted in Figure 31. Although the Ly108 knockdown efficiency only reached 25%, expression and IL-4 production, compared to scrambled siRNA (Figure 31). In an alternative approach, we performed a similar set of experiments using a Ly108-Fc fusion protein that has been previously documented to block any trans-Ly108 interactions between BMDCs and iNKT cells (Figure 31) [333]. By preventing Ly108 interactions, we were able to recapitulate the previously observed decrease in CD69 expression as well as reduced IFN-γ and IL-4 production by iNKT cells (Figure 31). Together, these data show that Ly108 expression on both lipid-presenting cells and iNKT cells is critical to iNKT cell activation.

**Loss of Ly108 in B6.NZB(171-177) mice results in defective signaling leading to ineffective Erg2 and PLZF induction**

Other groups working with the NZM-derived Sle1b mice have identified a skewing of Ly108 splice variants with increased expression of Ly108-1 and decreased expression of Ly108-2. As the Ly108-1 isoform is more heavily phosphorylated than the Ly108-2 following engagement, this has been shown to lead to enhanced Ly108 signaling [263]. However, there are a number of reported polymorphisms between NZB and NZW mice in Slamf6 and our strain has a distinct and previously unreported loss of iNKT cell function. We therefore examined expression of Ly108 splice variants in thymocytes and BMDCs by qRT-PCR to determine if B6.NZB(171-177) mice had a similar skewing. In contrast to Sle1b mice, B6.NZB(171-177) mice had a slight reduction in all splice
variants and a significant loss of *Ly108H-1* in both thymocytes and BMDCs (Figure 32). To assess whether this loss affects Ly108 signaling in B6.NZB(171-177) mice, we stimulated pre-selection DP thymocytes with immobilized anti-CD3 antibodies alone or in combination with stimulatory anti-Ly108 antibodies, and measured upregulation of the transcription factors Erg2 and PLZF at 2 and 48 hours post-activation, respectively [333]. As expected, Ly108 co-activation potentiated TCR-mediated expression of Egr2 and PLZF in B6 pre-selection DP thymocytes. Consistent with impaired Ly108 signaling in B6.NZB(171-177) mice, this potentiation was significantly reduced in B6.NZB(171-177) pre-selection DP cells (Figure 32).

To confirm the role of Ly108 signaling in iNKT cell activation, we also sought to examine activation of freshly isolated iNKT cells. Unfortunately, Ly108 expression levels are altered by activation, and therefore experiments examining freshly isolated or *in vitro* expanded primary iNKT cells could not be performed. As an alternate approach, we co-cultured Ly108 expressing iNKT hybridomas with αGalCer-pulsed B6 BMDCs and measured activation by IL-2 accumulation in overnight cultures. Similar to what was observed in our *in vivo* experiments with DPs, expression of Ly108 significantly enhanced activation leading to increased cytokine production (Figure 32). Together, these findings suggest that Ly108 co-stimulation potentiates TCR-mediated iNKT cell activation, and that the loss of Ly108 expression in B6.NZB(171-177) mice leads to ineffective signaling and stimulation.
Figure 32: Loss of Ly108 leads to ineffective PLZF and Erg2 upregulation in pre-selection DP Thymocytes.

(A) Expression of Ly108 splice variants as measured by qRT-PCR from total thymocytes and rhFLT3L cultured BMDCs. (B) Erg2 mean fluorescence intensity measured in pre-selection double positive thymocytes after two hours of stimulation with plate bound anti-CD3 and -Ly108. (C) PLZF positive cells were measured after 48 hours of culture following stimulation of pre-selection DP thymocytes with plate bound anti-CD3 and Ly108. (D) IL-2 production was measured after overnight co-culture of Ly108+ or Ly108- NKT cell lines with B6 BMDCs pulsed with various concentration of αGalCer. Data is representative of 4 independent experiments. Each data point represents an individual mouse with bars representing population medians. Data in A-C was analyzed by Mann-Whitney U tests comparing B6 and B6.NZB(171-177) groups. * p < 0.05, ** p < 0.01, *** p < 0.001. Data in D was analyzed by Two-Way Anova.
Ly108 expression and signaling is critical to human iNKT cell activation and correlates with peripheral iNKT cell frequencies.

Alterations in iNKT cell numbers and function are observed in patients with autoimmunity and cancer, however, the connection between Ly108 expression and signaling in these cells has yet to be defined. To address this question, we examined Ly108 expression on peripheral blood mononuclear cells (PBMCs) from healthy individuals. As expected, Ly108 was expressed by all peripheral immune populations with the highest levels of expression observed on iNKT cells (Figure 33). Of note, T cells had a bimodal distribution of Ly108 expression which segregated by CD45RA expression (Figure 33), suggesting that similar to mice, activated T cells upregulate expression of Ly108 after stimulation, as previously reported [342].

To determine the impact of Ly108 on iNKT cell function, PBMCs were incubated in vitro with αGalCer in the presence of stimulatory anti-Ly108 or isotype control antibodies. Cytokine production by iNKT cells was measured by flow cytometry. The addition of anti-Ly108 antibody clearly and robustly increased both TNF-α and IFN-γ production by iNKT cells (Figure 33). Given the normal variations in the levels of Ly108 in healthy control populations, we asked whether Ly108 expression correlated with iNKT cell frequencies. As previously observed in mice, peripheral iNKT cell frequencies positively correlated with Ly108 expression on CD45RA-negative lymphocytes (Figure 33). This suggests that variations in Ly108 expression may also control iNKT cell development and/or peripheral homeostasis in humans. In further support of a role for
Ly108 in iNKT cell activation in humans, the levels of Ly108 on iNKT cells correlated positively with IFN-γ production after stimulation with αGalCer (Figure 33). Thus, Ly108 may play a similar important role in control of iNKT cell numbers and function in humans as observed for mice.
Figure 33: Ly108 signaling in human iNKT cells augments activation.

(A) Ly108 expression on PBMC subsets. Values represent the mean and standard error from 7 healthy controls. Significance as compared to total lymphocytes was determined by Kruskal-Wallis and Dunn’s multiple post-tests. (B) Representative plot of Ly108 expression versus hCD1d-PBS57 tetramer. (C) Representative plot of Ly108 versus CD45RA on T cells (CD3⁺CD19⁻). (D) Example gating of rested PBMCs stimulated overnight with DMSO or 100 ng/ml αGalCer. NKT cell (Tet⁺CD3⁺CD19⁻) activation was measured by intracellular cytokine accumulation. NKT cells were gated as CD19⁻CD3⁻hCD1d⁺PBS57 Tetramer⁺. (E,F) IFNγ and TNFα production as measured by flow cytometry after overnight culture with αGalCer in the presence of varying concentrations of isotype control (blue) or anti-Ly108 (red) antibody. Bars show mean and standard error. (G) Median fluorescence intensity of Ly108 on CD45RA⁻ lymphocytes correlates with NKT cell frequencies in the blood. (H) Frequency of IFNγ producing NKT cells from with αGalCer stimulated PBMCs correlated with median fluorescence intensity of Ly108 on NKT cells. PBMCs were profiled from 7 healthy male controls. Each point represents an individual control. Data with three or more groups was analyzed by paired non-parametric Friedman test with post-tests compared to basal stimulation.
Discussion

In the present study, we have unraveled a key role for Ly108 homotypic interactions in the activation of mouse and human iNKT cells. Using our unique NZB congenic mouse strain, we mapped defects in iNKT cell development and function to the polymorphic NZB Slam locus, which is associated with consistently reduced expression of Ly108, in multiple cell types including DP thymocytes, iNKT cells and dendritic cells. Using knockdowns and blocking strategies, we showed that Ly108-Ly108 interactions between DCs and iNKT cells are critical for optimal peripheral iNKT cell activation and cytokine secretion. We further demonstrate that the reduced levels of Ly108 in B6.NZB(171-177) mice are associated with markedly attenuated DP thymocyte signaling and that Ly108 is required for optimal signaling in an iNKT cell line. Finally, we provided evidence that Ly108 plays a similarly important role in human iNKT cell development/homeostasis and activation.

We have previously reported that iNKT cell frequencies are reduced in B6 congenic mice with an NZB chromosome 1 interval extending from 133-177 Mb and that genes on NZB chromosome 4 can partially correct for this loss but do not restore iNKT cell function [74]. Here we show that this loss of splenic iNKT cell frequency and function is entirely recapitulated in B6.NZB(171-177) mice carrying a small NZB chromosome 1 interval which contains the Slam locus. Previous work has suggested that there are 2 broad Slam haplotypes which can be used to subdivide inbred strains of mice and that NZW (Sle1b), NZB, and 129 mice all share the same haplotype [241]. However,
with the advance of sequencing technology, it has become apparent that even within mice of the same haplotype, there are multiple sequence differences within the *Slam* locus. Of particular note and relevant to the current study, within *Slamf6*, there are 133 single nucleotide differences between NZB and NZW strains located primarily in the regulatory upstream and downstream regions (http://www.sanger.ac.uk/). With this mind, it is not surprising that there may be differences in iNKT cell phenotype and function between strains with the same *Slam* haplotype. Although in general there is a paucity of information regarding iNKT cells in Sle1b mice, a slight reduction in the frequency of splenic iNKT cells has been reported [227]. This finding contrasts with evidence that Ly108 signaling is increased in DP thymocytes from this strain leading to increased expression of transcription factors that would be expected to enhance iNKT cell differentiation in the thymus [333]. Similar changes in the expression of *Ly108-1* and *Ly108-2* isoforms have also been demonstrated for congenic mice with the 129 *Slam* allele and are associated with increased proportions of thymic (but not splenic) iNKT cells, and slightly reduced function of splenic iNKT cells [343]. In contrast to the Sle1b and 129 alleles, the NZB *Slam* allele is associated with reduced levels of *Ly108-1*, *Ly108-2*, and *Ly108-H1* that results in markedly attenuated signaling in DP thymocytes, impaired thymic iNKT cell development, and a greater than 60% reduction in the capacity of these cells to secrete cytokines. Thus, study of the NZB *Slam* allele provides a unique opportunity to assess the impact of these molecules in iNKT cell development and activation.
As outlined previously, the 171 to 177 Mb region on NZB chromosome 1 is the minimal interval required for anti-nuclear antibody production, suggesting that the Slam locus plays a critical role in the generation of this phenotype. It is currently unclear whether the altered iNKT cell subsets and function in these mice plays a direct pathogenic role, as there are multiple functional abnormalities in a variety of immunologic populations. For example, B6.NZB(171-177) T cells, which also have a drastic loss of Ly108 are hyper-responsive to TCR engagement, leading to enhanced Th1 differentiation and proliferation [68,334]. B6.NZB(171-177) B cells also demonstrate signaling defects, however in this case attenuated Ig receptor signaling leads to impaired induction of B cell tolerance mechanisms (Cheung et al, submitted). In contrast to the findings for iNKT cells, these functional changes are similar to those seen for Sle1b mice, highlighting that different immune populations may be differentially sensitive to polymorphisms in the Slam locus.

Cell specific regulation of SLAM receptors, their isoforms, and adaptors likely leads to distinct effects on various immune cell populations. This is further complicated by polymorphisms in the Slam locus, which can disrupt this complex regulation. This is highlighted in the present study where we assessed Ly108, CD150, and Ly9 levels on a variety of immune cells and found marked changes in the levels of expression of these molecules in B6.NZB(171-177) relative to B6 cells. For example, we noticed that the expression of CD150, Ly108, and Ly9 from cultured BMDCs was not identical to the pattern in ex vivo DCs isolated from spleen. These differences indicate the importance of
assessing SLAM expression in the precise populations that are being examined and calls into question studies where inferences regarding function were made without assessing SLAM expression. In the case of Ly108, this is further compounded by differences in the signaling capacity of the different isoforms, which could lead to altered signaling despite similar levels of cell surface expression. Additionally, there is little known about how these different isoforms of Ly108 interact with the various intracellular adaptors to mediate function.

Unlike studies of Ly108 and CD150 gene deleted mice, investigations of mice with natural variations allows for the examination of physiologically relevant changes and their impact on disease pathogenesis. Here we show that a genetic polymorphism that results in a ~80-90% decrease in expression on DPs is sufficient to alter thymic iNKT cell development, recapitulating the findings seen with the Ly108 knockout[262]. Given the role of CD150 and CD1d in thymic NKT cell development, it is possible that the less dramatic changes in CD150 (which was reduced 10-20%) and CD1d (which was reduced ~50%) also contributed to the impaired iNKT cells in these mice. The mechanism leading to reduced CD1d expression on B6.NZB(171-177) DP thymocytes is currently unknown since the CD1d gene is not located within the NZB interval and there are no obvious genes within this interval that are predicted to affect lipid presentation. It is possible that the altered SLAM signaling in these cells leads to impaired upregulation of CD1d, however this will require further investigation. Nevertheless, our findings clearly indicate
that naturally occurring genetic polymorphisms have a significant impact on iNKT cell biology.

In the presented work, iNKT cell function was initially assessed by intravenous injection of αGalCer. Recent studies have shown that activation of iNKT cells after injection of glycolipid is dependent on marginal zone dendritic cells and patrolling iNKT cells [344,345]. It has been suggested that intravenous αGalCer injections preferentially activate iNKT1 cells with vascular access in the red pulp of the spleen and liver [213]. However, splenic iNKT2 were also activated, but to a lesser degree [213]. As iNKT1 cells can also produce IL-4 to some extent, our initial observation with αGalCer injections showing impaired activation of both IFN-γ and IL-4-producing iNKT cells, may have uncovered a defect in the DC-iNKT activation interface specific to iNKT1 cells rather than defects in both iNKT1 and iNKT2 cells. This may explain why the relative increase in iNKT2 cells did not translate to increased IL-4 production.

With the obvious skewing in NKT cell subsets, frequency, and development, it is somewhat surprising that injection of αGalCer-pulsed Ly108 competent B6 BMDCs could fully recapitulate activation of iNKT cells in B6.NZB(171-177) recipients. This finding suggests that there is dissociation between iNKT cell developmental defects, steady-state cytokine production, and activation potential. Although subsets of iNKT cells may be indicative of steady state cytokine production [212], this appears to be modulated by enhancing or interfering with SLAM receptor co-signaling. This raises the
possibility that the functional losses observed in a number of mouse models may be unrelated to iNKT developmental defects but rather due to the loss of co-stimulation at the DC-iNKT cell interphase in the periphery.

It has been suggested that Ly108 signaling may function in cis, acting directly on the cell surface of one cell or in trans, between two interacting cells. In NK cells, for instance, a recently published report has shown that NK cell education may occur through cis interactions on NK cells[346]. Our data supports the idea that for iNKT cells, trans-Ly108 signalling plays an important role in their activation; however, the action of cis interactions cannot be completely ruled out. In fact, cis signaling on iNKT cells may explain how the transfer of Ly108 deficient BMDCs into wild type B6 animals could result in normal activation of iNKT cells.

As we uncovered an important role for Ly108 in iNKT cell activation in mice, we set out to confirm our results in humans. To our knowledge, no direct association between Ly108 and iNKT cell frequency and function has been identified in humans. We show for the first time that in healthy controls, Ly108 expression is highest on peripheral iNKT cells and that cross-linking Ly108 in the presence of αGalCer increases iNKT cell activation. The high expression of Ly108 in iNKT cells may be related to their overall memory/activated phenotype, as seen in T cells [272]. We also show that, as in mice, there are positive correlations between Ly108 expression and iNKT cell frequencies and function in healthy controls. At present, the genetic basis for variations in iNKT cell
expression in the human population is unknown. While reduced levels of iNKT cells are seen in the family members of patients with SLE, it is unknown whether these changes are genetically mediated or arise from a more general immunologic disturbance as indicated by the association with anti-nuclear antibodies. Although the Slam locus has not been identified in genome wide association studies of SLE, two recent publications have found higher expression of NTB-A (Ly108) in SLE T cells with an associated increase in signaling and Th17 frequencies [272,273]. While this seems discordant with the reported decreases in iNKT cell frequencies in SLE, it is likely that it results from the increased activation of T cells observed in this condition.

In summary, this work has highlighted an important and novel role of Ly108 in the function of mouse and human iNKT cells. Importantly, through BMDC transfer experiments we have dissociated the confounding effects of Ly108 on iNKT cell development from its role in iNKT cell activation. The growing body of literature on Slam family proteins suggests that these molecules, although important for T-B cell interactions, may play a large and underappreciated role in the innate immune response.
CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

The goal of the research presented in this thesis has been to use a combination of congenic lupus-prone mice and knockout animals to decipher the genetics of autoimmunity. Unlike the other described models of SLE, congenic animals, like the ones used in this research, allow for the precise study of the genetics that contribute to spontaneously arising autoimmunity, and thus are likely to be more reflective of the human pathogenesis. In the presented research, that work has focused exclusively on deciphering the genetics of New Zealand Black mice and the contribution of these loci to autoimmunity, B1a, and NKT cell development and function.

The foundation for this thesis was based on the original observation that bicongenic B6.NZBc1c4 mice had a suppressed autoimmune phenotype with expanded B1a and iNKT cell compartments, as compared to B6.NZBc1 mice. Using previously generated congenic mice, as well as by generating novel shorter interval congenic and knockout animals, the suppressive role of CD5⁺ B cells was elucidated and the limited role of iNKT cells uncovered. Chapter II demonstrated that CD5⁺ B cells and not iNKT cells from B6.NZBc4 mice were likely suppressive in bicongenic B6.NZBc1c4 mice. Using adoptive transfer techniques, it was shown that the frequency of pro-inflammatory T cell populations can be reduced by transfer of CD5⁺ B cells from B6.NZBc4 mice. Noting that CD5⁺ B cells had increased production of IL-10, in Chapter III, the exact role of this suppressive cytokine was studied using IL-10 knockout animals. Unexpectedly, it
was shown that IL-10, although critical for suppression of autoimmunity in B6.NZBe4 animals, was also vital to the homeostatic expansion of innate-like populations including CD5+ B cells, iNKTs, and NK cells. Lastly, given the literature on iNKT cells and their ability to drastically influence the immune response, it was investigated why the expansion of iNKT cells from B6.NZBe1c4 had minimal impact on disease. In Chapter IV, it was discovered that iNKT cells from NZB chromosome 1 animals are defective in function and this was retained in B6. NZBe1c4 animals. This may explain their relative unimportance in the suppression observed in these mice. This defect was further localized to a loss of Ly108 expression, caused by NZB SLAM receptor polymorphisms, at the DC-iNKT cell synapse.

As mentioned in the introduction, the study of regulatory B cells is still largely hindered by a lack of a defined ontogeny. The absence of a unifying critical transcription factor has led to the hypothesis that regulatory B cells can arise from a number of different B cell populations. In the case of B6.NZBe4 mice, the expanded CD5+ B cell compartment in the spleen was noted to have a suppressive effect. Given the expansion of peritoneal B1a cells, it is tempting to assume that this general expansion is directly responsible for the increase of splenic CD5+ B cells. As a highly IL-10 competent population, it is possible that in our mice, these regulatory B cells are somehow directly associated with the B1a cell population. However, preliminary experiments with transfer of peritoneal B cells failed to recapitulate the observed losses in pro-inflammatory T cell subsets. It is possible that autoreactive/regulatory CD5+ B cells had migrated from the
peritoneum cavity and localized to the spleen, explaining these differences. We hypothesize that the expansion of CD5+ splenic B cells is unique to our animals. This is supported by the differences in antigen specificity of splenic CD5+ B cells between strains.

The above findings suggest that splenic CD5+ B cells from B6.NZBc4 mice are somehow distinct. In Chapter III, this is further validated by the observation that expansion of CD5+ B cells may not be necessary to completely protect against autoimmunity. Studies with B6.NZBc1c4t mice revealed that suppression of disease can occur regardless of CD5+ expansion. As was repeatedly suggested throughout the thesis, the presence of receptor editing or other genes may promote the selection of regulatory B cells in NZB mice. Studies from NZM-derived animals have identified the receptor editing defects on both chromosome 1 and chromosome 4, some of which may be shared with NZB animals. It may be possible, then, that like regulatory T cells, B cells can acquire this phenotype through self-reactivity in the absence of overt help. Thus, genes that promote the expansion of B1a cells and limit their repertoire towards autoreactivity may be generating regulatory B cells in the spleen, where autoantigens are encountered. Future studies on these mice should focus on deciphering the differences between B6.NZBc1c4 and B6.NZBc1c4t mice, both capable of suppressing disease when compared to parental B6.NZBc1 mice. A detailed understanding of BCR usage and repertoire between CD5+ B cells and other splenic B cell populations could yield valuable functional insights into the development of regulatory B cells. Furthermore, tracking
studies examining the movement of splenic versus peritoneal B cell populations could be undertaken in B6.NZBc4 mice to identify if these cells populations are related and whether migration and/or retention occurs in response to autoantigens.

The role of IL-10 in B cell survival is well established and was validated in our studies with congenic mice in Chapter III. As many studies with regulatory B cells rely on total or B cell specific IL-10 knockouts, our findings raise the possibility that autocrine production of this cytokine is critical to function. This calls into the question some of the earlier work with regulatory B cells. If the lack of IL-10 prevents these regulatory B cells or B1a cells from surviving, this can have confounding effects on studies of B cell transfers. Alternatively, it may be possible that this reliance only comes about because of the forced homeostatic expansion of these populations in our congenic mice. It could be that a deficiency in cell-cycle inhibitor genes, such as the previously described reduction in p18, creates increased reliance on IL-10 in maintaining this increased turnover. To unravel these problems, future studies will need to identify how these pathways may be interacting through in vitro culture or cell-specific double knockouts.

One key feature of the chromosome 4 congenic mice used in this thesis was the expansion of splenic, hepatic, and thymic iNKT cells. Interestingly, these expansions have not been reported in similar Sle2 congenic mice. The exact genetic basis leading to this expansion was not investigated in this work, but it is interesting to note that Jak1 lies
within the minimal interval associated with this expansion and that a SNP polymorphism does exist in this gene between NZB and NZW mice (http://www.sanger.ac.uk/). Jak1 lies downstream of IL-15, a known homeostatic regulator of NKT cell frequency in mice and humans [347–349]. It may be possible that hyperactive IL-15 signaling is responsible for this homeostatic expansion and should be investigated in future studies. In fact, this polymorphism may also explain the increased numbers of NK cells which are regulated by IL-15 signaling [350]. Similarly, a study showed that mucosal B1 cells proliferate in response to IL-15 and differentiate into IgA producing B cells [351]. Additionally, IL-10 signaling is also downstream of Jak1 and some studies have shown that inhibition with this cytokine required Jak1 signaling. This raises the possibility that enhanced IL-10 signaling due to a Jak1 polymorphism may also feedback to allow for greater development of regulatory B cells. Clearly, Jak1 function should be researched in future studies to identify any SNP-related differences and their related effects on the homeostatic expansion and differentiation of NKT and B1a cells.

The discovered functional and development defects in NZB chromosome 1 mice raise some critical questions about the need for SLAM receptors in maintaining iNKT cell frequencies. As B6.NZBc1c4 mice, which have both the chromosome 4i NKT cell expansion and the chromosome 1i NKT cell developmental defects, retain their expansion of splenic iNKT cells. Preliminary work from B6.NZBc1c4 mice revealed similar defects in SLAM receptor expression in the thymus and periphery and functional defects in iNKT cell responses. How the chromosome 4 mediated expansions can
override the critical need for SLAM receptor signaling is largely a mystery and suggests that iNKT cell development and/or peripheral homeostasis is much more complicated than previously appreciated. Indeed, development and peripheral homeostasis may be two entirely different mechanisms under the control of different regulatory networks. Future studies will need to uncover this interplay further.

Since iNKT cells recognize glycolipid presented on CD1d and subsets of regulatory B cells have been reported to have increased CD1d expression, it is possible, that these two populations may directly interact. In fact one recent study has shown that CD1d knockout animals have a reduction in regulatory B cells, suggesting a possible link [352]. In support of this hypothesis, another study showed that the activation of iNKT cells resulted in the recruitment and activation of regulatory B cells [353]. From our studies, however, the expansions of these two populations were entirely independent of one another. In Chapter II, knockout of CD1d had no impact on the frequency of CD5+ B cells or IL-10 production. Similarly, when we transferred B cells or CD5+ B cells, the proportion of iNKT cells was unchanged. Control of these expansions, therefore, seems to be entirely genetic and localized to the c4 91-123 Mb interval. To further dissociate these populations, conditional knockout of CD1d on B cells should be undertaken along to explore these interactions in our congenic animals.

The role of SLAM receptor signaling in autoimmunity and iNKT cell development has been well-established within the last half-decade. Seminal works have
shown a clear need for SAP signaling in T-B cell interactions and induction of the iNKT cell lineage. However, most of these works have highlighted the critical role of SAP and not the role of individual SLAM family receptors. With 9 family members and multiple isoforms and splice variants, the relative role of individual SLAM receptors has been difficult to discern. On top of this, there may be some overall redundancy in function between the family members. Using knockout animals, work has begun to tease apart the individual roles of these molecules, highlighting discernible differences.

Although the impact of polymorphisms in the SLAM locus on development of murine lupus is well established, there is still much work to be done to determine the individual Slam family member contributions to this immune pathogenesis. For instance, conflicting studies have shown both positive and negative roles for Ly108 signaling. These differences may arise from differential expression of signaling adaptors such as SAP or SHP-1 and vary by cell type and inflammatory cues. As an example, during germinal center responses between T and B cells, Ly108 expression may be critical to recruiting SHP-1 to the synapse in order to dampen BCR signaling and allow for effective antigen capture and costimulation. However, in the periphery, T-DC interaction may provide positive co-stimulation through Ly108 in order to drive a robust immune response. The same SLAM family receptor may therefore have entirely different outcomes depending on the stimulation conditions and interacting cell type. In Chapter IV, it was discovered that NZB-derived animals have an absence of Ly108 expression
leading to a significant loss in TCR-mediated stimulation. How the exact isoforms of Ly108 play into this dynamic needs to studied further in future studies.

Collectively, this thesis has shown that CD5⁺ B cells and not iNKT cells may suppress autoimmunity in B6.NZBc1c4 mice. The genetics of these expansions and suppression of autoimmunity was resolved through generation of subcogenic mice and IL-10 knockouts. Finally, using these same unique strains, a reliance on Ly108 signaling in iNKT cell activation was discovered, possibly explaining their limited involvement in our disease models. From this work it is clear that the use of congenic mice to decipher the genetics of autoimmunity and their contribution to innate lymphoid cells still holds promise. Deciphering these genetics components using these tools can lead to a better understanding of disease and ultimately help to develop novel therapeutics for SLE patients.
REFERENCES


36. Kawasaki A, Tsuchiya N, Fukazawa T, Hashimoto H, Tokunaga K. Analysis on the association of human BLYS (BAFF, TNFSF13B) polymorphisms with


48. Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK. Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and


82. Gay D, Saunders T, Camper S, Weigert M. Receptor editing: an approach by


155. Scalapino KJ, Tang Q, Bluestone JA, Bonyhadi ML, Daikh DI. Suppression of


199. Liu TF, Jones BM. IMPAIRED PRODUCTION OF IL-12 IN SYSTEM LUPUS ERYTHEMATOSUS. II: IL-12 PRODUCTION IN VITRO IS CORRELATED NEGATIVELY WITH SERUM IL-10, POSITIVELY WITH SERUM IFN-γ AND NEGATIVELY WITH DISEASE ACTIVITY IN SLE. Cytokine. 1998;10: 148–153.


201. Yuan W, DiMartino SJ, Redecha PB, Ivashkiv LB, Salmon JE. Systemic lupus


258. Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled


289. Matsushita T, Horikawa M, Iwata Y, Tedder TF. Regulatory B cells (B10 cells) and regulatory T cells have independent roles in controlling experimental autoimmune encephalomyelitis initiation and late-phase immunopathogenesis. J Immunol. 2010;185: 2240–2252.


305. Cai G, Kastelein RA, Hunter CA. IL-10 enhances NK cell proliferation,


COPYRIGHT ACKNOWLEDGEMENTS

- Chapter I: Introduction
  - Figure 4 & Figure 3 reproduced with permission from Cannons JL, Tangye SG, Schwartzberg PL (2011) SLAM family receptors and SAP adaptors in immunity. Annu Rev Immunol 29: 665–705. doi:10.1146/annurev-immunol-030409-101302.

- Chapter II: Suppression of autoimmunity by CD5+ IL-10-producing B cells in lupus-prone mice
  - A link can be found at: http://www.nature.com/gene/journal/v16/n5/full/gene201517a.html

- Chapter III: IL-10 Production is Critical for Sustaining the Expansion of CD5+ B and NKT cells and Restraining Autoantibody Production in Congenic Lupus-Prone Mice.
  - A link can be found at: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0150515