NZB/BINJ AND NZW/LACJ EMBRYONIC CHIMERAS DEVELOP STRONG AUTOIMMUNITY DEPENDENT ON NZB/BINJ T CELLS

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DEDICATION

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NZB/BINJ AND NZW/LACJ EMBRYONIC CHIMERAS DEVELOP STRONG AUTOIMMUNITY DEPENDENT ON NZB/BINJ T CELLS

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NZB/BINJ AND NZW/LACJ EMBRYONIC CHIMERAS DEVELOP STRONG AUTOIMMUNITY DEPENDENT ON NZB/BINJ T CELLS

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The NZB/NZW F1 hybrid develops systemic lupus erythematosus (SLE), displaying features of human disease including spontaneous anti-nuclear antibodies, glomerulonephritis, earlier and more penetrant expression of disease in females, and polygenic etiology. The autoimmunity that develops in these mice must result from epistatic interactions between NZB and NZW alleles at specific loci in the hybrid mice. However, the causative alleles and pathways have remained elusive. In the present study we sought to determine whether

incompatibility between cells of the two parental strains causes autoimmunity. We generated chimeras (here designated NZB;NZW) by injecting embryonic stem cells from NZB/BINJ (NZB) mice into NZW/LacJ (NZW) blastocysts. These chimeras developed an accelerated form of autoimmunity characterized by the presence of autoantibodies 3 months earlier, and at titers 10-fold greater, than the NZB/NZW F1 hybrids. The chimeras also developed mild glomerulonephritis and severe lymphadenopathy and splenomegaly. The observed cellular incompatibility was specific for the NZB;NZW combination (not observed in NZB;C57BL/6J or NZW;C57BL/6J), and occurred despite the fact that each parental type does not develop SLE features in a non-chimeric environment. Within each chimera, an expansion of activated T cells from the NZB strain and a predominance of B cells from NZW were observed. Furthermore, the expanded NZB T cells correlated with autoimmunity, and removing these T cells in NZB$^{Cd3e-/-}$;NZW chimeras prevented disease. Thus, NZB and NZW cells are inherently incompatible with one another, though compatible with C57BL/6J cells. Pathogenic intercellular transactions cause this strong autoimmunity in chimeric mice, dependent upon the presence of NZB T cells.

TABLE OF CONTENTS

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Randesi, M., O. Levran, J. Correa da Rosa, J. Hankins, J. Rule, M.J. Kreek, W.M. Lee, A. Reuben, R.J. Fontana, T. Davern, B. McGuire, R.T. Stravitz, V. Durkalski, I. Liou, O. Fix, M. Schilsky, D. Ganger, R.T. Chung, D. Koch, K.R. Reddy, and L. Rossaro. 2017. Association of Variants of Arginine Vasopressin and Arginine Vasopressin Receptor 1A With Severe Acetaminophen Liver Injury. *Cellular and Molecular Gastroenterology and Hepatology* 3:500-505.

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LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF DEFINITIONS

SLE – Systemic Lupus Erythematosus

ESC – Embryonic Stem Cell

NZW – New Zealand White (LacJ specifies Jackson Lab Strain)

NZB – New Zealand Black (BINJ specifies Jackson Lab Strain)

ADCC – Antibody-dependent Cell-mediated Cytotoxicity

CLL – Chronic Lymphocytic Leukemia

AIHA – Autoimmune Hemolytic Anemia

GVHD – Graft-versus-Host Disease

RBC – Red Blood Cell

QTL - Quantitative Trait Locus

LN – Lymph Node

PB – Peripheral Blood

PF – Peritoneal Fluid

MZB – Marginal Zone B Cells

FoB – Follicular B Cells

CHAPTER ONE
Introduction

SLE is an autoimmune disease affecting many tissues and organs. It results largely from autoantibodies against nuclear antigens, including RNA, DNA, histones, and RNA-binding proteins. SLE autoantibodies can directly bind to cells to cause cell lysis, and can also form immune complexes with cellular contents, promoting vascular injury, rashes, and glomerulonephritis. In most cases the exact cause of SLE is unclear. However, it is known to be due to a combination of genetic and environmental factors [24, 233, 290]. Mutations at single loci may cause disease with high penetrance in both mice and humans [48, 228, 229]. However, most SLE cases may have a complex genetic etiology, insofar as known monogenic causes have not been identified. It is proposed that SLE develops in a stepwise manner. First there is a break in tolerance to self-antigens, leading to the generation of nonpathogenic autoantibodies. This autoreactivity can be amplified by aberrant interactions between T and B cells or dendritic cells, leading to the development of autoreactive effector cells and pathogenic autoantibodies. Finally, these may culminate in cell activity such as pathogenic cytokine release, autoantibody dependent cell cytotoxicity, complement deposition, and inflammation that causes disease in target organs [6, 137, 267, 298]. Like many other autoimmune diseases, SLE is more commonly observed in women, who develop the disease 9 times more often than men [115]. Studies have identified feminizing hormones, mainly estrogen, as pathogenic contributors to SLE in murine models. However in humans this pathogenic effect is less clear [138, 246].

NZB/NZW F1 hybrid mice develop a disease that closely resembles human SLE. These animals spontaneously develop anti-nuclear autoantibodies at 5-6 months of age, with maximal levels observed at 6-8 months, leading to renal disease [285]. Quantitative trait mapping has implicated regions of genomic DNA from the NZW parental strain (including *Sle1*, *Sle2*, and *Sle3*) in specific aspects of SLE pathogenesis. However, definitive assignment of causation to allelic variants of specific genes is lacking in that each locus contains multiple genes that might potentially contribute to SLE [204]. By placing these regions onto the C57BL/6J background, the contribution of each region to the disease has been explored. *Sle1* has been shown to cause a breach in B cell tolerance, leading to the production of anti-nuclear antibodies [193]. The *Sle2* locus has been shown to result in an expanded autoreactive B1a population [192]. The *Sle3* region is required for glomerulonephritis but only in conjunction with *Sle1* [194]. Independent of one another these loci do not result in the complete SLE phenotype. Furthermore, loci outside of *Sle1*, *Sle2*, and *Sle3* and regions from the NZB strain are likely involved in disease but were not tested for their contribution. This is due to reliance on the recombinant inbred NZM2410 strain in mapping studies which has 76.3% of the genome derived from NZW [202].

Throughout all studies of the NZB/NZW F1 hybrid mice, there has been an implicit and well-warranted assumption that dominant contributions from each strain participate in causing the disease phenotype. The fact that a recombinant inbred line develops SLE indicates that a certain combination of loci bearing homozygous alleles of NZW and NZB origin are pathogenic as well. However, it has never been clear whether individual nuclei must be

programmed with a pathogenic genotype. Does SLE result strictly from an unlucky combination of alleles within all cells? Or would the genetic contributions of each strain be sufficient to cause SLE even if maintained within separate cells of the same animal? The question cannot be addressed by creating bone marrow chimeras, in which graft vs. host disease and graft rejection would obscure autoimmunity. Accordingly, we created NZB;NZW blastocyst chimeras, by injecting NZB embryonic stem cells (ESC) into NZW blastocysts. These mice are composed of NZB and NZW parental cells that have developed together within the tolerogenic environment of the embryo. Interestingly, they develop exaggerated and precocious autoimmunity, more severe than that seen in NZW/NZB F1 hybrids.

CHAPTER TWO
Review of the Literature

SYSTEMIC LUPUS ERYTHMATOSUS

Epidemiology and Diagnosis

Due to a diverse range of presentations and broad diagnostic criteria, calculating the incidence and prevalence of SLE has been difficult. In the U.S., the prevalence has been reported between 42-300 per 100,000 people and the incidence as 5.5-23.2 per 100,000 per year. This range can vary considerably in other nations. Due to both genetic and environmental components of the disease, different ethnicities have a higher prevalence, including African Americans and indigenous peoples of various nations[36, 115]. In addition, SLE has a strong female bias, reported to be approximately 9 times more prevalent in females than males[80, 283]. Sex hormones have been identified as an underlying factor, as women more frequently develop the disease during "reproductive years" between approximately 20-60 years of age[183, 230, 239].

There have been several revised criteria for diagnosing and monitoring SLE. The most recent guidelines for diagnosis include the American College of Rheumatology (ACR) 1997 and Systemic Lupus International Collaborating Clinics (SLICC) 2012 classification criteria[119, 227, 318]. The SLICC classification states a physician may "classify a patient as having SLE if he or she satisfies 4 of the clinical and immunologic criteria used in the SLICC classification criteria, including at least one clinical criterion and one immunologic criterion OR if he or

she has biopsy-proven nephritis compatible with SLE in the presence of ANAs or anti-dsDNA antibodies". Meanwhile, the ACR guideline requires at least 4 of the 11 criteria to be present for SLE diagnosis. Based on comparative studies, the SLICC guidelines have been shown to have higher sensitivity than the 1997 ACR criteria (Table 1)[111, 123]. Additionally, activity and chronicity indexes are used to monitor the disease over time and are especially useful in clinical trials. Activity indexes include the Systemic Lupus Erythematosus Responder Index (SRI), the British Isles Lupus Assessment Group Scale 2004 (BILAG), and the SLE Disease Activity Index 2000 (SLEDAI-2k), as well as others. Meanwhile the SLICC/ACR damage index is used to determine chronic damage in various organ systems[236].

Table 1: Diagnostic Criteria for SLE.

ACR 1997	SLICC 2012 Clinical Criteria
1. Malar Rash	1. Acute Cutaneous Lupus
2. Discoid Rash	2. Chronic Cutaneous Lupus
3. Photosensitivity – Skin rash from sunlight	3. Oral Ulcers
4. Oral Ulcers	4. Non-Scarring Alopecia
5. Non-erosive Arthritis –2+ peripheral joints	5. Synovitis – 2+ joints
6. Pleuritis or Pericarditis	6. Pleuritis or Pericarditis
7. Renal Disorder – Proteinuria >0.5mg/day or >3+ or cellular casts present	7. Renal Disorder – Proteinuria >0.5mg/day or >3+ or cellular casts present
8. Neurological Disorder – Seizures or Psychosis	8. Neurological Disorder – Seizures or Psychosis
9. Hematological Disorder – Hemolytic anemia or Leukopenia or Lymphopenia or Thrombocytopenia	9. Hemolytic Anemia
10. Immunological Disorder – Anti-dsDNA or Anti-Sm or Anti-Phospholipid antibodies	10. Leukopenia or Lymphopenia
11. Positive Antinuclear Antibody	11. Thrombocytopenia
	SLICC 2012 Immunologic Criteria
	1. ANA
	2. Anti-dsDNA
	3. Anti-Sm
	4. Antiphospholipid Antibody
	5. Low Complement
	6. Direct Coombs' Test

Adapted from [1] and [227]

Pathogenesis

Etiology

The etiology of SLE lies in the intersection of genes, epigenetics, and environmental factors. Single-gene mutations in the complement pathway are known to cause SLE in humans, including *C1q* and *C4*, as well as *Fas*, *Fasl*, *Trex1*, *Roquin* in mice. However, in general most SLE cases are polygenic and the exact genes involved are not known[228]. Heritability alone cannot fully account for all cases of SLE, as twin studies calculate an approximate 25% monozygotic concordance rate[59, 97, 293]. As a result, environmental factors must be involved, including those such as viral infection, epigenetics, sun exposure, and drug effects. In general, it is considered that most individuals who develop SLE have variant alleles at multiple loci that predispose them to autoimmunity, initially without overt disease. This is then exacerbated after exposure to pathogenic environmental influences[99, 196]. Finally, sex also affects autoimmunity, as women develop SLE 9 times more frequently than men[80, 283]. Studies have identified feminizing hormones as playing an important role in the sex bias of SLE in murine models. Work has shown this can be mediated by the estrogen receptor, particularly the α isoform, which acts as a transcription factor to influence cytokine and antibody secretion. However, the estrogen receptor is not the only contributing factor, and other influences, such as androgens or estradiol likely impact disease onset[138, 246].

Autoantibodies

As seen in the diagnostic criteria, there are several different disease manifestations in SLE, and it is possible two individuals may both be diagnosed with few overlapping symptoms. One hallmark of SLE is the production of IgG anti-dsDNA antibodies as well as antibodies with other specificities that can cause damage to organs throughout the body. In a recent study, it was found that these autoantibodies develop very early and pre-date the first clinical manifestation of disease by years[6]. However, many healthy individuals that never develop SLE also can produce these autoantibodies, especially anti-Ro and anti-La[101, 280].

Tissue Injury

Renal disease is commonly seen in SLE due to the activity of autoantibodies. These autoantibodies may cause tissue damage by directly binding to kidney antigens[64, 187, 321] or binding to nucleosomes from apoptotic cells, forming immune complexes found in the glomeruli[135, 136, 191]. These autoantibodies can induce damage through complement fixation and FCγ receptor signaling. The classical complement pathway (C1, C2, C4), is important in the clearance of cellular debris and is protective in SLE[128, 178]. However, the alternative complement pathway (C3, C5) can induce damage in the kidney by forming the membrane attack complex (MAC) and promoting pro-inflammatory cytokine release and cell migration[15, 178, 218]. FCγ receptors are also implicated in causing tissue damage. Multiple GWAS have identified the inhibitory receptor, FCγIIb and activating receptor, FCγIIIb as contributing to SLE[325]. Furthermore, genetic studies in SLE mouse models have also

implicated FcγRs (see Chapter Two "NZB/NZW F1 MODEL"). FcγRs have diverse functions. As a result, like complement, FcγRs can inhibit SLE by increasing apoptotic cell clearance and modulating lymphocytes, but also promote inflammation via antibody-dependent cell-mediated cytotoxicity (ADCC), cytokine secretion, or oxidative burst[212, 213]. FCγIIb is inhibitory to B cells, and deletion in B6 results in increased nephritis and autoimmunity[28], while overexpression in NZM2410 or MRL/*lpr* mice ameliorate autoimmune features[32, 189, 269]. On the other hand, FCγIII is an activating FCγR, and can lead to ADCC and inflammation[213]. Deletion of activating FCγR in NZB/NZW F1 led to a decrease in mortality and proteinuria, even in the presence of autoantibodies and immune complex deposition[50].

In addition to renal pathology, SLE can also cause tissue injury at other sites, including the CNS, vasculature, or in a developing fetus. Some anti-dsDNA antibodies can cross-react with *N*-methyl-d-aspartate receptor (NMDAR), a neural antigen, leading to neuronal cell death. This could contribute to the nervous system manifestations seen in some SLE patients. However, these antibodies must be able to breach the blood-brain barrier to enact their effects[7, 68, 106]. Furthermore, SLE patients may develop anti-phospholipid antibodies, although they are not sensitive or specific as clinical markers of SLE. These bind to a variety of neutral and anionic phospholipids and are associated with increased thrombotic events[51, 182, 221, 284]. On the other hand, anti-Ro and anti-La antibodies can traverse the placental barrier

and cross-react with fetal antigens. As a result, it is associated with fetal heart block and mortality[129, 265].

Common Pathways of Disease

Although SLE is generally polygenic and has diverse features, there are common aberrant pathways identified in patients with SLE and mouse models. These pathways include defective clearance of DNA from cell debris[179], immune complex uptake leading to increased TLR activation and type I interferon, and dysfunctional B and T cell activity[249].

Clearance of Cell Debris

Defective clearance of cell debris can lead to an abundance of autoantigen. This DNA and associated proteins can originate from cells undergoing apoptosis or neutrophil NETosis. Some individuals with SLE show a decrease in the removal of apoptotic cells[89], phagocytosis[117, 184], and NET degradation[103, 167]. Furthermore, knockouts or mutations in genes involving the breakdown of DNA, such as *TREX1*[96], *DNase-1*[2, 209], and *C1q*[29] leads to autoimmunity.

Increased Type-I Interferon and TLR Signaling

This debris is then taken up by phagocytes and antigen-presenting cells such as DCs, plasmacytoid dendritic cells (pDC), monocytes, and macrophages via FC receptors. In

response to immune complexes, DCs can be activated to increase antigen presentation and maturation[241]. Neutrophil NETs can be stimulated by autoantibodies and have been shown to activate pDCs and monocytes[40, 156]. Furthermore, immune complexes from apoptotic and necrotic cells can induce interferon α (IFN-α) production in pDC[177]. This activation occurs via endosomal TLR7 and TLR9. Indeed, inhibition of TLRs prevented IFN-α secretion after exposure to immune complexes[17, 190]. IFN-α is produced in large quantities in patients with SLE, and this can be correlated with disease severity[21, 317]. The increased production of IFN-α causes leukocytes to upregulate interferon-stimulated genes and leads to the interferon gene signature that is commonly seen in SLE[13, 22]. Some mouse models of SLE, including the NZB/NZW F1, have an interferon gene signature[326]. NZB/NZW F1 and NZB mice lacking the IFN-α receptors (IFNAR) or treated with TLR7/9 inhibitors have decreased autoimmune features[18, 253]. Meanwhile, overexpression of IFN-α increases disease severity[188]. However, other mouse models, including MLR/*lpr*, do not show an IFN-α phenotype[326]. The effect of IFN-α on leukocytes promotes many features of the disease (reviewed in [11, 43]). IFN-α signaling leads to increased antigen presentation and differentiation of DCs[26, 177], TH1 cell generation[124], and antibody production and class switching in B cells[161].

Aberrant T and B Cell Function

In addition to increased activation of the innate immune system, dysfunctional B and T cell activity is commonly found in autoimmune diseases. Both B and T cells must break tolerance in order to develop full SLE features, and defects at both peripheral and central tolerance

checkpoints have been identified in SLE patients and mouse models, however there are differences between mouse strains and individuals (reviewed in [252, 322]). For example, SLE patients and mice have increases in BlyS/BAFF[98, 226], a B cell survival factor that is important for peripheral B cell maturation, especially for self-reactive B cells[31, 169, 287].

B cells from SLE patients have been shown to have Lyn deficiency[174]. Lyn is both a positive and negative regulator in B cells and can phosphorylate CD22, FCγRIIb , SHP-1, and SHP-2 to reduce B cell activation[311]. Indeed, mice devoid of Lyn develop autoantibodies and glomerulonephritis[118].

T cells from patients with SLE have decreased expression of CD3ζ and increased expression of FcεFγ in TCR signaling. Since FcεFγ binds to Syk strongly after TCR engagement, this leads to increased calcium flux compared to the typical CD3ζ-ZAP70 signaling pathway[152]. Blocking Syk in MRL/*lpr* mice led to decreased splenomegaly, lymphadenopathy, skin lesions, and kidney disease, while in NZB/NZW F1 mice it increased survival, decreased the number of activated T cells, and reduced kidney disease[14, 61]. While in both strains disease features were ameliorated, it did not prevent autoimmunity. Expression of CD3ζ in SLE T cells prevents this aberrant signaling and results in normal levels of calcium flux after TCR stimulation[208]. Meanwhile, mice lacking CD3ζ have a multi-organ inflammatory phenotype but no autoimmunity[62]. Increased calcium flux ultimately results in increased CD40L and B cell help[155]. In addition to these changes in CD3 signaling, SLE T cells have been shown to

have an increased number of pre-formed lipid rafts, which decreases the threshold for T cell activation[153], and altered CREM/CREB regulation of IL-2[271]. Mouse models of SLE have greater levels of mTOR activation and cellular metabolism[315]. This promotes TH1 and TH17 differentiation, as well as CD4+ T cell activation and development into effector T cells. Modulating mTOR and glucose metabolism in SLE patients, *Sle1*.*Sle2*.*Sle3*.B6, and B6.*lpr* mouse models resulted in decreased autoimmune features[82, 315, 316].

Besides differences in signaling pathways, mouse models and patients with SLE have changes in overall frequency and function in B and T cells. TFH cells have a significant role in autoimmunity since they are vital in helping B cells produce class-switched antibodies. TFH are increased in some individuals with SLE[266] and overproduction of TFH can cause autoimmunity in mice (see "Sanroque")[173]. Furthermore, increased populations of IL-17 producing cells have been observed in patients with SLE, and this can correlate with disease[52]. SLE is a TH1-biased disease with enhanced IFN-γ secretion[109]. This IFN-γ can lead to greater glomerulonephritis, antibody secretion[102], TFH cell differentiation[165], and BlyS/BAFF secretion by monocytes[109].

Treatments

Current treatments for SLE include anti-malarials, glucocorticoids, and biologics. Anti-malarials, such as hydroxyquininone, can reduce SLE features by permeating cell

membranes, lysosomes, and vesicles. This leads to increased alkalinity in the lysosome which disrupts antigen presentation, TLR signaling, and cytokine secretion[86]. Glucocorticoids are broadly immunosuppressive and act via glucocorticoid receptors to affect gene expression and signaling pathways that reduce leukocyte migration and pro-inflammatory cytokine secretion[248, 274]. A more targeted approach was developed by utilizing belimumab, an anti-BLyS (BAFF) and rituximab, an anti-CD20. Both target B cell plasmablasts by inhibiting the B cell survival factors (BLyS) or the B cell receptor (CD20)[220]. However, these drugs have limited targets and either generally inhibit the immune system or B cells. Furthermore, glucocorticoroids and anti-malarials can have strong side effects leading to ancillary tissue injury. Fortunately, many novel therapeutics are currently being assessed in clinical trials, including those targeting cytokines, B , T, and dendritic cells, and interferons[57, 81].

NZB/NZW F1 MOUSE MODEL

Parental Strains

New Zealand White (NZW)

The NZW strain has the H-2z haplotype and does not develop autoimmunity. It is largely considered a healthy, inbred strain[286]. However, studies into NZW have shown some abnormalities. NZW NKT cells were found to have higher secretion of IL-4 and IFNγ compared to other inbred strains, such as B6 and Balb/C, with NZB producing negligible amounts[291]. They were found to have low quantities of FOXP3+ T cells. This was attributed to poor stability of the Treg phenotype outside of the thymus[65]. Furthermore, NZW mice are prone to mild nephritis and exhibit some renal defects upon aging with increased lipoprotein deposits in their kidneys[141, 217]. This can be made severe when challenged with anti-glomerular basement membrane serum, or bred with other strains of mice such as NZB, B6.*Yaa*, and B6.*Sle1*[116, 127, 202, 309].

New Zealand Black (NZB)

The NZB strain has the H-2d haplotype and possesses a variety of disease features. The mice develop chronic lymphocytic leukemia (CLL) and autoimmune hemolytic anemia (AIHA). The CLL is caused by proliferation of B-1 cells that are hyperdiploid[83, 238]. Studies were performed to identify genes associated with this disease, and regions were found on

chromosomes 14, 18, and 19 that are outside of previously identified autoimmunity-associated loci[237]. The region on chromosome 14 is related to the human 13q14 locus, which is well known to be involved in CLL[34, 70]. *MiR15* and *miR16* lie within this region. MicroRNAs bind to mRNA to cause degradation or prevent translation[19]. The target of *miR15/16* is *BCL2*, which aids in cell survival and is commonly upregulated in CLL and other cancers in humans and the NZB strain[49, 55, 250]. As a result, deletions or mutations of these microRNA lead to increased *BCL2* expression and decreased apoptosis[49]. Due to these discoveries, BCL-2 inhibitors have been recently developed for a variety of cancers and venetoclax has been approved for patients with CLL[8, 224].

The AIHA in the NZB mice present with IgM anti-nuclear and anti-RBC antibodies[120, 286]. The main epitopes of the anti-RBC antibodies are against band 3 protein, as well as other components of RBC, which are also the main epitopes in human AIHA[16, 58, 104, 162]. The cause for their AIHA is unknown. There have been several studies performed to elucidate this mechanism, however no specific genes have been identified[144, 147, 163, 219, 222]. One possible mechanism is through an elevation in reactive oxygen species. As RBC carry oxygen, they can develop oxidative damage through the release of molecules such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2)[132]. The damaged molecules could then act as antigens in an immune response[148]. NZB mice have elevated levels of reactive oxygen species in their RBC[125]. In one study, eliminating SOD1, an enzyme that converts superoxide to hydrogen peroxide to remove free oxygen radicals, led to increased mortality, reactive oxygen species,

and oxidative damage of RBC. Meanwhile, overexpressing the enzyme decreased all of these features and IgG bound onto the surface of the RBC[148]. Furthermore, removing SOD1 from B6 mice or injecting them with oxidized RBC lead to the development of anti-RBC antibodies, while adding an anti-oxidant with the oxidized RBC did not[125].

In addition to these disease features, NZB mice also have a large population of marginal zone B cells (MZB) present in their spleens[9]. As well, it has been reported that NZB bone-marrow derived macrophages have greatly reduced inflammasome function. The cells have increased levels of p202, which represses AIM2 function, and a splice-site mutation in NLRP3 results in very low levels of the correctly spliced protein[262]. However, the relationship of these findings to autoimmunity has not been determined.

Disease Features of NZB/NZW F1

The NZB/NZW F1 mouse model was the first characterized spontaneous SLE model, and has since been studied for decades to identify the etiology and pathogenic mechanisms of disease[116]. NZB/NZW F1 mice develop glomerulonephritis, which is the main cause of mortality. They develop heavy mesangial proteinaceous deposits and proliferation of glomerular cells with crescent formation. As a result, these mice have large amounts of protein in their urine, at twice the quantities of MRL/*lpr* or BXSB mice. IgG and C3 deposits can be seen via immunofluorescence in the kidneys, especially in mesangial regions. IgG2a is the main isotype observed in the kidneys[268]. NZB/NZW F1 produce anti-dsDNA

antibodies, as well as other anti-nuclear antibodies, at approximately 5-6 month of age in females, and at 7-8 months in males. Furthermore, they develop splenomegaly and lymphadenopathy at approximately 8 months of age. All features develop earlier and with greater severity in females[286]. However, NZB/NZW F1 mice do not develop the skin lesions typical in some SLE mouse models.

SLE SUSCEPTIBILITY/RESISTANCE GENES

The NZB/NZW F1 develop disease based on genetic influences from the two parental strains. In many mouse models, such as BXSB/*Yaa* and MRL/*lpr*, the main genetic contributors to the disease phenotype are known. However, all the genes involved in NZB/NZW F1 have not been identified. In order to identify the genetic contribution of each strain, multiple studies have been performed on backcrosses of NZB/NZW F1. Mice are inbred for specific lupus phenotypes, and the genes are mapped. Several studies have been performed that recognized different loci of interest.

H-2

In almost every study performed looking for linkage of different alleles to SLE in mice, the H-2 locus is identified, regardless of the design taken[149, 200, 245, 302]. This locus corresponds to the MHC II locus in humans, which has the highest degree of linkage to SLE in multiple

GWAS analysis[121, 281]. Although it has such an important role in the disease, its mechanism is still unknown. In the NZB/NZW F1, disease is promoted with heterozygosity of the NZB (H-2^d) and NZW (H-2^z) alleles, while homozygosity of either is protective (*Sles1*)[150, 297]. Different transgenic mice have been generated looking at individual genes within this region, however no contribution to the disease was found.

NZM2410

Multiple backcrosses of NZB/NZW F1 yielded the inbred NZM2410 strain that was selected for highly penetrant SLE[247]. This was crossed to B6 mice to search for loci related to autoantibody production and glomerulonephritis[200]. Using this model, four main quantitative trait loci (QTLs) were identified, *Sle1*, *Sle2*, *Sle3*, and *H-2*, and they were bred into congenic strains to isolate their function[201]. More analysis broke each QTL into smaller segments, each with different phenotypes. To determine protective alleles, NZW mice were crossed with B6.*Sle1*/NZW F1 and mice with SLE features were selected.

Sle1 (Telomeric Chromosome 1, NZW Origin)

Genes in this region cause the development of autoantibodies such as anti-chromatin and anti-histone. However, mice congenic for this region do not develop glomerulonephritis[193]. This region can further be broken down into *Sle1a, 1b,* and *1c*, with *Sle1b* accounting for most of the phenotype[203]. Within *Sle1b* are SLAM/CD2 family genes

(*Cd48, Cd150, Ly108, Cd84*) as well as non-SLAM/CD2 family genes (*Usp23, Nit1, Refbp2, Ncstn, Copa, Pxf*), and different genes within this region have shown effects on autoimmunity[204]. One of the main candidate genes is *Ly108*, however it alone cannot account for the whole phenotype[143, 299]. The NZW *Ly108* led to decreased B cell deletion and anergy, possibly allowing self-reactive B cells to escape into the periphery[154]. More recently, it was found to be involved in the expression of PLZF, a transcription factor necessary for innate T cell and NKT cell development[73]. *Sle1a* can be further broken down into smaller loci, including *Sle1a1* and *Sle1a2*[53]. *Sle1a1* contains only one gene, *Pbx1*. This gene has a single mutation that leads to increased expression of the *Pbx1-d* isoform. *Pbx1-d* acts as a dominant negative by competing with *Pbx1-b*, another isoform which maintains chromatin inactivation[261]. Expression of this isoform in Jurkat cells led to increased activation and reduced activation-induced cell death. Furthermore *Sle1a1* results in autoreactive T cells, a reduction in Foxp3+ Tregs, increased TFH, and an upregulation of many microRNA and CD44[45, 54, 216]. *Sle1c* has a mild phenotype leading to small levels of autoantibody, however it likely promotes disease in epistasis with other QTLs. Within this region is *Cr2*, which encodes complement receptors 1 and 2. A mutation in this gene causes increased glycosylation which likely reduces binding to its ligand, C3d, or prevents dimerization. This leads to reduced activation in B cell responses to IgM cross-linking in the context of Cr1/2 signaling. However, this gene has yet to be confirmed as the cause for this phenotype[27]. The *Sle1c* QTL can be divided into *Sle1c2*, which accounts for an autoreactive CD4+ TH1 cell phenotype. Within this region is *Esrrg*, which encodes for ERRγ, estrogen-related receptor γ,

that regulates mitochondrial function. As T cell metabolism has been increasingly appreciated for its role in T cell activation and differentiation, it is possible this gene promotes glycolysis and thus TH1 differentiation. However this has not been confirmed.

Additionally, within the *Sle1* region is *Fcgr2b*, an inhibitory B cell Fc γ receptor. Expressing higher quantities of *Fcgr2b* in the NZM2410 strain prevented the SLE-like phenotype, while removing it from B6 (but not Balb/c) caused autoimmunity and glomerulonephritis[28, 189]. When the NZW allele of *Fcgr2b* allele was placed in the B6 strain, it did not cause autoimmunity but led to decreased expression of *Fcgr2b* in germinal center B cells and more IgG class-switching after immunization[234]. As a result, it is possible that *Fcgr2b* contributes to the autoimmune phenotype in conjunction with other genes. Furthermore, the *Fcgr2b* gene in NZB mice was also linked to autoimmunity in the *Nba2* locus described below.

Sle2 (Chromosome 4, NZW and NZB Origin)

Sle2 is unable to cause nephritis, but induces B cell hyperactivity with increased IgM production and B-1 cell populations[192]. *Sle2* has been broken down into 3 smaller regions, *Sle2a* (from NZW*)*, *Sle2b* (from NZW*)*, and *Sle2c* (from NZB*)*, with *Sle2c* contributing the most to the phenotype. Within *Sle2c* lies *Cdkn2c*, cyclin-dependent kinase inhibitor p18INK4c (p18), responsible for regulation in the cell cycle. The NZB allele of *Cdkn2c* causes a four-fold decrease in expression, as well as in other surrounding genes. This inhibitor causes G1 arrest, and in B6.*Sle2c* mice, B-1 cells underwent greater proliferation.

B-2 cells, on the other hand, had different phenotypes, because their proliferation and renewal is regulated differently[273, 289, 312]. Indeed, specifically knocking out p18 in B6 mice lead to increased B-1a populations and even autoantibody production[232]. Another gene in this region is *FAAH*, fatty acid amide hydrolase. Mice with the NZB allele have about 5-fold rise in expression and have increased enzyme function in the spleen. Upon inhibition of this enzyme in NZB and *Sle2* mice, the phenotype was reversed, with decreased B-1 populations and IgM production. The mechanism by which FAAH affects B cells however has been undetermined[223]. *Sle2a* from NZW caused increased migration of cells to the marginal zone in the 3H9/56R transgenic model, however the mutation responsible for this phenotype has not be identified[320].

Sle3 (Chromosome 7, NZW Origin)

Individually *Sle3* is unable to cause nephritis or autoantibodies, but leads to increased activated CD4+ T cells in the LN and spleen. Combining *Sle1* and *Sle3* leads to extension of the antibody repertoire to include anti-dsDNA and anti-glomerular basement membrane antibodies and glomerulonephritis[194, 195]. For the nephritis phenotype, candidate genes within this region include *Klk*, which are serine proteases the regulate many different pathways, including inflammation, and are known to be involved in renal and cardiovascular disease[42, 175]. Expressing *Klk* in B6.*Sle3* mice challenged with anti-GBM antibodies showed improvement in renal function[171]. Meanwhile, transferring *Klk* expressing mesenchymal stem cells into 129/svj mice challenged with anti-GBM antibodies improved their nephritis as well

[172]. More recently, *Klk1* was overexpressed in B6.*Sle1*.*Sle3* mice under an inducible promoter. Mice induced to express *Klk1* had decreased proteinuria, creatinine, BUN, and renal inflammation as well as improved glomerulonephritis scores[263]. As a result, it is possible part of the contribution of the *Sle3* locus is enhanced renal inflammation in response to autoantibodies initiated by *Sle1*. For the T cell hyperactivation phenotype, it was found this is DC-intrinsic and T cell-extrinsic[270]. *Sle3* DCs were more proinflammatory and costimulatory. When they were treated with LPS and injected into B6, the *Sle3* phenotype of increased CD4+ activated T cells and low level autoantibodies appeared[324]. However, the genes involved in this process have not been identified.

NZM2328

When intercrossing NZB/NZW F1 mice, both NZM2410 and NZM2328 mouse strains were generated. NZM2328 has unique female-biased glomerulonephritis (GN) phenotypes of both acute and chronic GN. However not all mice developed autoantibodies. As a result, it serves as a better model to understand genetic factors involved in kidney disease. Acute kidney disease is defined as only mesangial proliferation and glomerular hypercellularity, whereas chronic kidney disease included sclerosis, tubular atrophy/dilation, and interstitial fibrosis[302].

Cgnz1 and Agnz1 (Chromosome 1, NZW origin)

Unlike the work completed in NZM2410, researchers studying NZM2328 introduced WT C57L/J genomic regions onto the NZM2328 strain to see if the phenotype could be altered. Replacing the whole region containing *Agnz1* and *Cgnz1* on chromosome 1 led to a decrease of both acute and chronic GN[303]. However, when only the *Cgnz1* region was replaced, the mice developed acute GN and never progressed into chronic GN. This region overlaps with *Sle1b*. At this time, no causative gene(s) have been specifically identified for these regions[92].

NZB/NZW F1 x NZW Backcross

In addition to inbreeding NZB/NZW F1, one group backcrossed the NZB/NZW F1 onto NZW to try to identify NZB genes involved in SLE. These studies complemented the work done in the NZM strains, as they consisted mostly of NZW genome, with NZM2410 being approximately 76.3% from NZW[202]. Through the analysis of this backcross, *Nba2* and *H-2* were found to be linked to disease[72, 245].

Nba2 (Chromosome 1, NZB origin)

Interestingly, *Nba2* overlaps with the *Sle1* locus; however, each arose from a different parental strain. Mice congenic for the NZB *Nba2* locus have increased autoantibody production, increased B cell activation, and do not develop proteinuria unless crossed to NZW mice. Although these congenic mice have B cell abnormalities, they do not have the expanded MZB or B-1a populations typically seen in NZB/NZW F1 and NZB mice[9]. Within

this region are the *SLAM/CD2* family genes, *FcγR*, and *Ifi200* family genes, which are all thought to interact to induce the phenotype, similarly to *Sle1*. To test the different effects of these genes subcongenic strains of Nba2-A (*FcγR*), Nba2-B (*SLAM/CD2*), and Nba-C (*Ifi200*) were generated on the B6 background. Nba2-A (*FcγR*) and Nba2-B (*SLAM/CD2*) resulted in the formation of different subsets of autoantibodies, while Nba-C (*Ifi200*) had no autoimmunity independently. Combining the strains to create Nba2-AB resulted in the full spectrum of autoantibodies seen in B6.Nba2 mice. When isolated, the Nba2-A (*FcγR*) locus led to decreased *FcγRIIb* on B cells and less apoptosis, which was confirmed to be due to polymorphisms in the promoter region[310]. Furthermore, the Nba2-B (*SLAM/CD2*) locus showed a higher frequency of CD19+ pDCs, which could promote autoimmunity through secretion of IFN[134]. Although Nba-C (*Ifi200*) was not sufficient to cause autoimmunity alone, it is possible it regulates *FcγR* and SLAM/CD2 in the context of IFN stimulation[46].

NZB/NZW F2 Intercross

Similar to the other studies, NZB/NZW F1 mice were bred together to identify genomic regions of interest. In these studies, genes relating to splenomegaly, glomerulonephritis, and autoantibody production were characterized[149].

Lbw2 (Chr. 4, NZB origin)

NZB/NZW F1 mice carrying homozygous or heterozygous alleles of NZB or NZW *Lbw2* were found to have variations in their disease phenotype. Mice homozygous or heterozygous for the NZB allele have early mortality, splenomegaly, severe glomerulonephritis, and greater IgM levels. Mice homozygous for the NZW allele have decreased mortality and glomerulonephritis [108]. Placing the homozygous *Lbw2* locus from NZW into the NZB strain attenuated anti-RBC IgM responses, splenomegaly, and decreased MZB and B-1 populations down to B6 levels. However did not prevent anti-nuclear antibody production[259]. No candidate genes have been identified within this region, however based on the previous literature, *Lbw2* likely modulates IgM production in NZB. *Lbw2* is in a similar region as *Sle2*, however *Sle2* consists of both NZB and NZW genome. The portion of *Sle2* from NZB has been identified to regulate B-1 populations, similar to *Lbw2*[259].Similar findings were also found by Loh, *et al*. upon investigation of NZB chromosome 4[176].

SLE Resistance Genes

Notwithstanding the identification of these many NZB and NZW SLE susceptibility loci, it has always been implicitly clear that the parental strains must also express genes that prevent SLE, as they do not normally develop the disease. For example, B6 mice containing NZW alleles for *Sle1* and *Sle3* develop autoantibodies and glomerulonephritis, however wildtype NZW mice do not.

Sles1 (Chr. 17, NZW)

B6 mice containing the NZW chromosome 1 (*Sle1*) were backcrossed onto NZW to identify genes that could suppress the phenotype[202]. From this method *Sles1-4* were recognized, however only *Sles1* has been characterized further. *Sles1* completely prevents the development of autoantibodies associated with *Sle1*. This has been narrowed down to a 956kbp region containing the MHC locus and excluding the TNF locus. However, no specific causative mutation(s) have been implicated[275].

Sle2c2 (Chr. 4, NZB)

This QTL was discovered upon dissection of the *Sle2* locus. To test its suppressive capabilities, B6 mice with *Sle2c2* were challenged with cGVHD. B6.*Sle2c2* mice were able to induce cGVHD, but could not maintain the response and had significantly less lymphocyte activation and autoantibody production compared to B6. By creating different bone marrow chimeras, it was discovered that the *Sle2c2* mediates its suppressive function through non-T, non-B bone-marrow-derived cells[313]. This mapped to *Csf3r*, the G-CSF receptor. In NZB mice there is a mutation in exon 10, and expression levels are not affected. B6.*Sle2c2* mice have decreased G-CSF binding to its receptor and decreased neutrophil recruitment after G-CSF stimulation. Administration of G-CSF to cGVHD-induced B6.*Sle2c2* prevented the suppressive function and led to normal cGVHD features of autoantibody production. B6 mice with *Sle1*, *Sle2*, and *Sle3* recapitulate all the features of the NZM2410 strain but also possess this NZB suppressive locus. Administration of G-CSF induced accelerated IgG

autoantibody production approximately 2 months earlier than mice without this treatment. Thus, it is suspected that *Sle2c2* causes reduced G-CSF binding leading to decreased pro-inflammatory neutrophil recruitment to prevent cGVHD[159].

Table 2: Summary of Characterized QTLS in the NZB/NZW Model

QTL	Location and Origin	Phenotype	Candidate Gene	Function of Gene
Susceptibility Loci				
H-2	Chr.17			
Sle1	Chr. 1	Anti-chromatin and anti-histone autoantibodies		
Sle1a1	NZW	Reduction in Foxp3+ Treg, increased TFH, increased CD4+ T cell activation, increased autoreactive T Cells	*Pbx1*	Dominant negative isoform that competes with Pbx1-b, to upregulate various genes (CD44, miRNA)
Sle1a2	NZW	Increased CD4+ T cell activation	N.D.	
Sle1b	NZW	Anti-chromatin and anti-histone autoantibodies	SLAM/CD2 family (*Ly108*?)	Multiple effects from different genes in this region.
Sle1c1	NZW	Mild autoantibody production	*Cr2*?	More glycosylation that may interfere with dimerization/ligand binding to reduce B cell activation
Sle1c2	NZW	Autoreactive CD4+ T cells, TH1skewed T cell population	*Esrrg?*	Related to mitochondrial cell metabolism functions and possibly T cell activation/differentiation
Fcgr2b	NZW	Increased IgG class switching	*Fcgr2b*	Decreased expression of *Fcgr2b* in germinal center B cells
Sle2	Chr. 4	B cell hyperactivity with increased IgM production and B-1 cell populations		
Sle2a	NZW	Increased migration of cells to the marginal zone	N.D.	
Sle2b	NZW		N.D.	
Sle2c	NZB	Increased B-1 cell population	*Cdkn2c*	Decreased expression of p18, an inhibitor of the cell cycle.
			FAAH	Increased expression of *FAAH*.
Sle3/Sle5	Chr. 7, NZW	Increased activated CD4+ T cells in the LN and spleen. DC-intrinsic; Increased costimulation	N.D.	

Sle3	NZW	Causes nephritis when combined with *Sle1*	*Klk*	Serine protease involved in inflammation
Nba2	Chr.1, NZB	Autoantibody production, hyperactive B cells	*FcgR* *SLAM/CD2* Family *Ifi200* Family	Decreased expression of *Fcgr2b* in B cells causes decreased apoptosis SLAM/CD2 led to increased CD19+ pDCs Ifi200 may have a role upon IFN stimulation
Cgnz1	Chr. 1, NZW	Chronic glomerulonephritis, severe proteinuria, female biased	N.D.	
Agnz1	Chr. 1, NZW	Acute glomerulonephritis	N.D.	
Lbw2	Chr. 4, NZB	Splenomegaly, glomerulonephritis, hyper IgM production, increased B-1 and MZB populations	N.D.	
Resistance/Suppressor Loci				
Sles1	Chr. 17, NZW	Completely suppresses Sle1-mediated autoantibody production	H-2 region	
Sle2c2	Chr. 4, NZB	Suppresses cGVHD response	*Csf3r*	Decreased binding of G-CSF to receptor leads to diminished neutrophil recruitment

N.D. = Not Determined

ADDITIONAL MOUSE MODELS OF DISEASE

Overview

Due to the heterogeneity of features during presentation and the multitude of genetic and environmental causes of SLE, there are many mouse models available to study[39, 225, 251, 286]. None of the strains completely recapitulate all of the features seen in humans, however each strain can be used to study specific aspects of the disease. Some strains that are induced to develop SLE must be given an external stimulus initially, while others develop it spontaneously. Induced strains allow the investigation of early initiating events, while spontaneous strains highlight genetic factors.

MRL/*lpr*

MRL/*lpr* mice carry an insertion in *Fas*, which causes non-functional gene transcripts to be produced [48]. This results in lymphoproliferation (lpr), characterized by an accumulation of double negative CD4-CD8- T cells in the lymphoid organs. Normally, as T cells develop in the thymus, self-reactive cells undergo apoptosis. However, *Fas* in rendered non-functional in these mice, preventing the apoptosis of lymphocytes through the binding with FasL [301, 304]. Correspondingly, mutations in *FasL* produce a similar phenotype, called generalized lymphoproliferative disorder (gld) [279]. Transferring *lpr* from MRL mice to other inbred strains showed that this can induce lymphoproliferation and early mortality, but not

glomerulonephritis. This indicates that the MRL background is important for all the features of disease, and mice that are not predisposed to kidney disease are refractory [142].

MRL/*lpr* mice exhibit common features of autoimmunity, including autoantibody production against DNA and nuclear antigens, glomerulonephritis, and severe lymphoid hyperplasia[5]. However, these mice also have cutaneous, arthritic, and neurological components that can be present in SLE but are not always see in other mouse models [93, 105, 131]. On the other hand, male and females are affected equally, unlike in humans [286].

Aberrant Fas signaling in humans also induces a similar disease, autoimmune lymphoproliferative syndrome (ALPS). Similar to MRL/*lpr*, individuals with ALPS have elevated populations of CD4-CD8- T cells and symptoms appear equally in men and women early in life. Individuals present with lymphadenopathy and splenomegaly and often progress to some forms of autoimmunity, such as autoimmune hemolytic anemia, but usually do not develop nephritis or other autoimmune features[170, 282].

BXSB/*Yaa*

BXSB/*Yaa* mice have a translocation from the telomere of the X chromosome onto the Y chromosome, which doubles the expression of multiple genes. These genes include *Tlr7*, which plays a large role in the phenotype[229, 254, 276]. Transferring *Yaa* onto the B6 background with the lupus-promoting *Sle1* locus or modulating *Tlr7* expression, demonstrated the

positive relationship of TLR7 function to autoimmunity[60, 79]. Due to the fact the translocation occurs on the Y chromosome, the phenotype is predominately found in males. The mice typically develop glomerulonephritis, autoantibodies, and lymphoid hyperplasia[5, 286]. Unlike some other mouse models, the BXSB/*Yaa* model also has the interferon-gene signature typically found in humans[12, 244]. Furthermore they have a characteristic reduction in the marginal zone B cell population and an increased population of monocytes[3, 307].

Similar to the BXSB/*Yaa* mice, human males with XXY or XX were found at higher frequencies in the SLE population than the healthy population, indicating a possible dose-dependent effect of the X chromosome[69]. Higher gene copy numbers of *Tlr7* were also found in individuals with childhood-onset SLE, but were not significant in other populations studied[90, 140]. TLR7 function in both males and females has also been implicated in human disease[37, 38, 197]. Variants have been shown to be associated with autoantibody production and interferon activity, including a SNP especially found in males[139, 166, 264]. In addition, variants in IRF5, which signals downstream of TLR7, have consistently and strongly been associated with SLE (reviewed in [63, 160, 197]) .

Sanroque

In a forward genetic screen to test for suppressors of autoimmunity in B6 mice, a Sanroque mouse strain was generated with a mutation in Roquin-1[296]. This protein is a RING-type ubiquitin ligase that represses many genes, including *ICOS*, which encodes a costimulatory

molecule necessary for T cell activation, T cell help, and antibody class switching [71, 180, 277]. The M199R mutation in these mice leads to increased *ICOS* expression, an accumulation of TFH cells, and large numbers of germinal centers. This eventually leads to autoantibody development, lymphadenopathy, splenomegaly, and glomerulonephritis[67, 173]. Interestingly, when Roquin-1 was knocked out completely, the phenotype differed, with early mortality and no autoimmunity detected. Furthermore, specifically knocking out Roquin-1 in the hematopoietic lineage only, showed elevated *ICOS* expression, spontaneous germinal centers, and splenomegaly, however no autoimmunity[23]. As the M199R mutation in the gene has no defect in mRNA binding, it is possible the mutation specifically disrupts other interactions that ultimately overcome the survival deficit in Roquin-1 knockout mice and allow the presentation of autoimmunity[10]. A recent study explored the role of Roquin-1on the PI3K-mTOR pathway[78]. Furthermore, IFN-γ production downstream of aberrant Roquin-1 was identified as an important driver of disease[165]. More investigation is required to better understand the mechanism by which Roquin acts in SLE, however this model demonstrates that excessive T cell help can drive germinal center reactions that yield autoantibodies and SLE-like autoimmunity[260].

B6 x 129/Sv

B6;129/Sv chimeras were regularly utilized in the early development of the blastocyst microinjection method. B6 blastocysts were used as the host and the 129/Sv ESCs were

genetically modified, since they were able to be cultured easily. However, after breeding the chimeras back onto the B6 background, the mice developed autoantibody production and mild glomerulonephritis. Based on these findings, it was identified that the B6 x 129/Sv hybrid was predisposed to autoimmunity, and that it was not due to the genetic modifications that were being performed in the experiments. Using QTL analysis, genes within chromosome 1 from the 129/Sv strain were found to be associated with disease (*Sle16*), with the region encompassing several loci already known to play a role in autoimmunity (*Sle1, Nba2*)[33, 35, 113]. Additionally, *Sle18* was identified on B6 chromosome 3 that could induce autoantibody production when placed the 129/Sv background. This locus also falls within the same region as other known disease modifying loci, including *Sles3*[114].

Pristane

In this model, mice do not spontaneously develop disease but require intraperitoneal injections of pristane (2,6,10,14-tetramethylpentadecane, TMPD), typically found in mineral oil. Initially pristane-injected BALB/c and SJL were identified to develop autoantibodies and glomerulonephritis, however many inbred mouse strains can also be induced to have an SLE phenotype. Depending on the strain, different varieties of autoantibodies will be produced[255, 256, 257, 258]. More recently, humanized mouse models and primates were observed to gain SLE features when injected with pristane, with primates developing cutaneous lesions typically unseen in mice[100, 300]. Pristane-induced mice also have arthritis, hemorrhagic pulmonary

capillaritis, and an interferon gene signature[47, 168, 207]. Pristane acts as an adjuvant to create strong inflammatory responses[206, 240]. Upon injection, it forms plasmacytomas and lymphoid neogenesis (ectopic lymphoid tissue) in the peritoneum, that ultimately leads to SLE-like features[231]. This model is dependent upon type I interferons and TLR7 signaling, and the cytokines IL-6, IL-12 and IFN-γ have an important role in disease pathogenesis[164, 207, 242, 243]. While this model is artificial and is not likely a cause of human SLE, it is useful in understanding initiating events, as cell activity can be monitored immediately after induction, as well as the interferon and cytokine signals that promote disease.

Parent-into-F1 Model (Chronic Graft vs. Host Disease (CGVHD))

The CGVHD model is another induced SLE mouse model. Two strains of different haplotype are bred to yield an F1 hybrid, and one of the parental strains cells are transferred into the semi-allogeneic F1 recipient. Due to the donor T cells' recognition of the non-allogeneic MHC on the F1, they form a response that results in GVHD. Depending on the response that is generated, different phenotypes can be observed. If CD8+ T cells dominate, they will attack MHC disparate cells, which lead to acute GVHD, lymphopenia, organ damage, and early mortality at 2-4 weeks. On the other hand, if CD4+ T cells mount a response, there will be an expansion of lymphocytes resulting in polyclonal B cell activation and SLE features, called CGVHD. The acute GVHD or CGVHD outcome is dependent upon the strains used or cells transferred, as CD8+ T cell responses will dominate CD4+ responses unless the strain

has a CD8+ T cell defect or those cells were removed before injection into the F1[75, 94, 272, 295]. Mice induced in this manner can develop anti-nuclear and anti-RBC antibodies, glomerulonephritis, and skin lesions, with females possessing more severe symptoms in some strains[85, 158].

The CGVHD model is more similar to SLE than the human condition of CGVHD after bone marrow transplant[319]. While this model is artificial, it elucidates pathways that can initiate SLE. Mice with normal B cell repertoires can be induced to develop anti-nuclear antibodies with sustained help from non-autoreactive T cells. This illustrates how T cells can initiate autoimmunity at the early stages of the disease, and further investigation is required[295].

EMBRYONIC CHIMERAS

Embryonic, or primary, chimeras occur when there is greater than 1 genetically distinct cell line present in an organism before organogenesis. This occurs between zygotes of 2 different fertilization events, either through fusion or injection of embryonic stem cells (ESC). Since this occurs prior to organ development, both the cell lineages are found throughout the tissues of the body. Secondary, or transplantation chimeras, occur when tissues are transferred after organogenesis between a donor and host of different genotypes, such as in organ transplantation. In this case, the different cell lineages are segregated in different organs[74, 186]. Organisms with trace quantities of cells of another genotype are microchimeras,

which can occur naturally between a fetus and their mother, or between multiple fetuses in the womb[25, 145, 181, 294].

Chimeras have proven to be very useful in a variety of fields (reviewed in [74, 186, 308]). For developmental purposes, the differentiation capabilities of various cells can be interrogated, such as to determine the origin of cell lineages, pluripotency competency, or cell migration. Genetically, it can reveal the roles of non-cell autonomous embryonic lethal mutations, cell autonomous vs non-cell autonomous effects, and allow complementation within tissues[74]. Furthermore, some interspecies chimeras can be generated, which could ultimately be used to make humanized organisms[308]. For the NZB;NZW chimeras used in this project, the chimeric environment overcomes the barriers of MHC incompatibility, since tolerance is induced at embryonic stages. Furthermore, it allows for isolation of the cell-autonomous functions of NZB and NZW in the SLE phenotype.

Table 3: SLE Mouse Model Characteristics

Mouse Strain	Cause of Disease	Disease Features
MRL/lpr	Etn insertion into *Fas* genes leading to non-functional gene expression.	Lymphadenopathy due to large populations of CD4-CD8- T cells. Glomerulonephritis, autoantibody production, arthritis, cutaneous kin lesions, neurological defects, early mortality at 5 months. Affects male and females equally.
BXSB/Yaa	Translocation of terminal region of X chromosome (Yaa) onto Y, leading to twice the expression of various genes, including *Tlr7*.	Glomerulonephritis, autoantibodies, lymphoid hyperplasia, monocytosis, reduced and MZB population. Mainly affects males.
Sanroque	M199R mutation in *Rc3h1* (Roquin-1) which causes the upregulation of various genes, including *ICOS*	Spontaneous germinal centers with high populations of TFH cells, splenomegaly, lymphadenopathy, glomerulonephritis
B6 x 129Sv	Hybridization of 129Sv and B6	Autoantibody production, slight glomerlunephritis
Pristane	Intraperitoneal injection of pristane	Autoantibodies (depending on strain), glomerulonephritis, arthritis. hemorrhagic pulmonary capillaritis, interferon gene signature.
Chronic Graft-vs-Host Disease	Non-allogeneic CD4+ response of injected parental T cells into F1 host	Autoantibodies (anti-nuclear, anti-RBC), glomerulonephritis, cutaneous lesions (in some strains), female bias (in some strains).
NZB/NZW F1	Hybridization of NZB and NZW	Glomerulonephritis, autoantibodies, lymphoid hyperplasia. Female bias.

T AND B CELL TOLERANCE

Thymic T Cell Tolerance Checkpoints

T cells undergo both central and peripheral tolerance mechanisms in order to prevent autoimmunity. Central tolerance occurs in the thymus as the developing thymocytes undergo positive and negative selection, through interactions with MHC expressing thymic epithelial cells and dendritic cells. The affinity of the TCR to the MHC governs the survival of the T cells, called the affinity hypothesis. TCR that have low recognition to self MHC can survive, while those that have strong affinity for self are deleted.

Positive Selection

During positive selection, double positive CD4+CD8+ (DP) T cells are tested for their ability to recognize self-MHC molecules. This recognition is necessary for T cell function, as a mature T cell must be able to bind to MHC-peptide complexes to create an immune response. Positive selection is mediated by cortical thymic epithelial cells (cTECs) that express MHC. The peptides presented by cTECs are distinctive[146]. MHCI peptides are made using β5t containing proteasomes, which are unique to cTECs and are required for normal positive selection[205]. Meanwhile, MHCII peptides are made through cTEC specific lysosomal proteins such as cathepsin L and thymus-specific serine protease (TSSP) after macroautophagy[30, 122, 146, 210]. DP cells that can bind to these MHC-peptide complexes signal

through their TCR and survive while the others that cannot die via apoptosis. Signaling through the TCR is regulated by proteins that influence the sensitivity and strength of the signal through mechanisms such as phosphorylation and dephosphorylation of the TCR[87], and calcium flux (reviewed in [91]). Furthermore, the affinity of the TCR is biased towards specific MHC molecules due to interactions of CDR regions of the TCR and the MHC[199, 314]. This enhanced TCR sensitivity and MHC-bias increases the likelihood for survival so a larger population of T cells can continue through the next stage of selection.

Negative Selection

After positive selection, T cells undergo negative selection in the thymic medulla as single positive T cells. Medullary thymic epithelial cells (mTECs) present tissue-restricted antigens (TRAs) to test the ability of the T cells to respond. Cells that respond strongly to self-peptides undergo apoptosis or develop into T regulatory cells to prevent peripheral autoimmunity[133, 215, 306]. mTECs are able to present TRAs from peripheral tissues through AIRE and Fezf2[4, 66, 278]. As well, thymic dendritic cells can present antigen and self-peptides to induce tolerance[112].

B Cell Tolerance Checkpoints

Like T cells, B cells also undergo positive and negative selection based on the signaling capability of the BCR. Positive selection ensures that the gene rearrangements have

generated a functional BCR that is expressed properly, while negative selection removes self-reactive B cells.

Positive Selection

During positive selection, only B cells with functional BCR survive and migrate from the bone marrow into the periphery. This survival is mediated by tonic signaling through the BCR via the Igα and Igβ chains[151, 198]. B cells with low or no IgM expression will continue to undergo gene rearrangements to make a functional BCR or ultimately die[292].

Negative Selection

B cells undergo negative selection in the bone marrow to remove autoreactive cells. Self-reactive B cells can either change their BCR specificity through receptor editing, be deleted through apoptosis, or undergo anergy. It is hypothesized that when the BCR signals strongly in response to self, the B cells downregulate IgM expression, which initiates RAG and further gene rearrangement, called receptor editing. However, receptor editing may also be initiated by strong signaling via BLNK[211]. If successful, this would save the B cell from deletion[288]. However cells that cannot rearrange their BCR to prevent autoreactivity will undergo apoptosis, or clonal deletion[44, 110]. This occurs through BIM and BCL-2 regulated apoptosis[76, 157]. Additionally, B cells can undergo anergy where they are autoreactive but unresponsive[77, 95]. These cells can exit into the periphery but have a short lifespan[88].

CHAPTER THREE

Methodology

BUFFERS AND MEDIA

Table 4: Composition of buffers and media.

Buffer/Media	Components
Lymphocyte Media	RPMI1640, 10% FBS, 100 U/ml Penicillin/Streptomycin, 2mM GlutaMax, 10mM HEPES, 0.1mM NEAA, 1mM Sodium Pyruvate, 0.1μM 2-Mercaptoethanol
R5 Buffer	RPMI1640, 5% FBS, 50 U/ml Penicillin/Streptomycin, 1mM Glutamax, 5mM HEPES
2i Serum-Free Media	50% DMEM/F12 with 1x N-2 supplement, 50% Neurobasal Media with 1x B-27 Supplement, 100 U/ml Penicillin/Streptomycin, 1mM Glutamax, 0.5mM Sodium Pyruvate, 0.1mM NEAA, 0.1mM 2-Mercaptoethanol, 1μM PD0325901, 3μM CHIR99021, 1000 IU/ml LIF
ES Media	High Glucose DMEM, 15% ES FBS, 2mM Glutamax, 100U/ml Penicillin/Streptomycin, 0.1mM NEAA, 1mM Sodium Pyruvate, 0.1mM 2-Mercaptoethanol, 1000 IU/ml LIF
KOMP Media	Knockout DMEM, 15% Knockout Serum Replacement, 4mM Glutamax, 100U/ml Penicillin/Streptomycin, 0.1mM NEAA, 1mM Sodium Pyruvate, 0.1mM 2-Mercaptoethanol, 1000 IU/ml LIF, 5μg/ml Insulin
MEF Media	High Glucose DMEM, 10% FBS, 2mM Glutamax, 100U/ml Penicillin/Streptomycin, 0.1mM NEAA, 1mM Sodium Pyruvate, 0.1mM 2-Mercaptoethanol

FLOW CYTOMETRY

Instrumentation

A BD LSR Fortessa SORP instrument was utilized to identify major cell populations. To sort cells of interest, a BD FACS Aria II SORP or BD FACS Aria Fusion was operated by the Children's Research Institute at UTSW. For all instruments, FACSDiva software was used to visualize and collect the data, and FlowJo was utilized to analyze the data.

Antibodies

The following are the fluorophore-conjugated antibody panels used for staining. All panels except the HSC panel include BD Pharmigen CD16/CD32 FC Block at 1/200 dilution.

Table 5. Flow cytometry staining panels.

** indicates intracellular staining required.

Thymus Panel			
Marker	**Fluorophore**	**Concentration**	**Clone**
MHCI (H-2kd)	PE	1/100	SF1-1.1
CD4	BV785	1/100	RM4-5
CD8	BV510	1/100	53-6.7
CD25	FITC	1/100	3C7
CD44	BV421	1/100	IM7
CD3 (TCRb)	APC	1/100	H57-597
CD69	PerCP Cy5.5	1/100	H1.2F3
B220	AF700	1/100	RA3-6B2

Hardy Fractions Panel			
Marker	**Fluorophore**	**Concentration**	**Clone**
MHCI (H-2kd)	PE	1/100	SF1-1.1
IgD	APC/Cy7	1/100	11-26c.2a
IgM	PerCP Cy5.5	1/50	R6-60.2
B220	FITC	1/100	RA3-6B2
BP-1	BV711	1/50	6C3
CD24	Pacific Blue	1/100	M1/69
CD138	BV605	1/100	281-2
CD43	APC	1/100	S7

HSC Panel			
Marker	**Fluorophore**	**Concentration**	**Clone**
Lineage Cocktail	AF700	1/5	17A2, RB6-8C5, RA3-6B2, Ter-119, M1/70
Sca-1	AF488	1/100	D7
CD16/32	PECy7	1/100	93
CD34	eFluor450	1/100	RAM34
CD127	PE-CF594	1/100	SB/199
CD117	APC	1/100	2B8
MHCI (H-2kd)	PE	1/100	SF1-1.1

FOXP3 Panel			
Marker	**Fluorophore**	**Concentration**	**Clone**
MHCI (H-2kd)	PE	1/100	SF1-1.1
CD4	BV785	1/100	RM4-5
FoxP3**	AF647 (APC)	1/100	MF23
CD3	FITC	1/100	145-2C11
CD25	BV421	1/100	PC61
CD44	PE-CF594	1/100	IM7

Peritoneal Panel			
Marker	**Fluorophore**	**Concentration**	**Clone**
CD19	FITC	1/200	1D3
B220	AF700	1/200	RA3-6B2
MHCI (H-2kd)	PE	1/200	SF1-1.1
CD43	APC	1/200	S7
CD5	BV421	1/200	53-7.3

Peripheral Immune Cell Panel			
Marker	**Fluorophore**	**Concentration**	**Clone**
CD5	BV421	1/200	53-7.3
CD8	BV510	1/200	53-6.7
CD11b	BV605	1/200	M1/70
CD45.2	BV650	1/100	104
CD11c	BV711	1/200	HL3
CD4	BV785	1/200	RM4-5
CD3	FITC	1/200	145-2C11
MHCI (H-2kd)	PE	1/100	SF1-1.1
CD44	PE-CF594	1/200	IM7
IgM	PerCP/Cy5.5	1/100	R6-60.2
NK1.1	PE-Cy7	1/200	PK136
CD43	APC	1/200	S7
B220	AF700	1/200	RA3-6B2
IgD	APC-Cy7	1/200	11-26c.2a

Follicular Panel			
Marker	**Fluorophore**	**Concentration**	**Clone**
MHCI (H-2kd)	PE	1/200	SF1-1.1
CD23	BV421	1/100	B3B4
CD21	PerCP Cy5.5	1/200	7E9
B220	AF700	1/200	RA3-6B2
CD3	FITC	1/100	145-2C11
Viability	Tonbo Ghost Dye v510	1/1000	
CXCR5	BV650	1/50	L138D7
PD1	BV711	1/100	29F.1A12
CD4	BV785	1/200	RM4-5

T Cell Subsets Panel			
Marker	**Fluorophore**	**Concentration**	**Clone**
MHCI (H-2kd)	PerCP Cy5.5	1/100	SF1-1.1
CD4	BV785	1/100	RM4-5
CD8	BV510	1/100	53-6.7
CD3	BUV395	1/100	145-2C11
IL-17**	PE	1/100	TC11-18H10
IFNgamma**	FITC	1/100	XMG1.2
IL-4**	APC	1/100	11B11

Ki-67 Proliferation Panel			
Marker	**Fluorophore**	**Concentration**	**Clone**
CD3	BUV395	1/200	145-2C11
CD4	BV785	1/200	RM4-5
MHCI (H-2kd)	PerCP Cy5.5	1/100	SF1-1.1
Ki-67**	PE	1/100	SolA15
Viability	Tonbo Ghost Dye v450	1/1000	
Stimulators	CellTrace FarRed (APC)	0.1μM	

Sample Preparation

Tissue Preparation

For organs such as LN, spleen, bone marrow, and thymus, mice were euthanized via CO_2 asphyxiation or isofluorane overdose and subsequent cervical dislocation. Tissues were collected under laminar flow and stored in 3% FBS in PBS on ice. For bone marrow, femur and tibia marrow was harvested by flushing the bone with 6ml of 3% FBS in PBS using a syringe. All the tissues were then disaggregated into a single cell suspension through a 40μm cell strainer in 6ml of 3% FBS in PBS using a syringe plunger. Samples were then centrifuged at 450xg for 5 minutes and the supernatant was removed via vacuum. If required, splenocytes were treated with 1ml RBC lysis buffer (SIGMA R7757) for 5 minutes at room temperature. It was then diluted in 4 ml of 3% FBS in PBS, centrifuged at 450xg for 5 minutes, and the supernatant was removed. Approximately 2 million cells were used for each sample for surface staining panels only. For intracellular staining, 5 million cells were used per panel due to cell loss throughout the procedure.

For blood samples, 100μl of blood was collected via submandibular bleeding into a tube with EDTA. Samples were centrifuged at 450xg for 8 minutes and plasma was removed.
For peritoneal fluid, 5ml of PBS was injected via the intraperitoneal route and rested for 1 minute. A small incision was then made in the abdominal cavity and the fluid was collected. The fluid was then centrifuged at 450xg for 5 minutes and the supernatant was removed.

Surface Staining

After tissue preparation, the samples were then resuspended in 50μl of 3% FBS in PBS and 50μl of the panel master mix (2x in 3% FBS in PBS) containing only surface antigen antibodies. If viability dyes were used, the sample was resuspended in PBS without FBS, and the panel master mix was prepared in PBS without FBS. The sample was then incubated for 30 minutes at 4°C protected from light. For organs cells, 1ml of 3% FBS in PBS was added and the suspension was centrifuged at 450xg for 5 minutes. The supernatant was removed and the pellet was resuspended in 200-300μl of 3% FBS in PBS for analysis on the instrument. For blood, 1ml of RBC lysis buffer (SIGMA R7757) was added and incubated at room temperature for 5 minutes. Samples were then pipetted through a 40μm filter to remove clots and 4ml of 3% FBS in PBS was added. The sample was centrifuged at 450xg for 5 minutes and then the RBC lysis was repeated. Afterward, the cell pellet was resuspended in 200-300μl of 3% FBS in PBS for analysis on the instrument.

Intracellular Staining

For panels requiring intracellular staining of FOXP3 and Ki-67, the BD Pharmigen Mouse Foxp3 fixation and permeabilization buffers were used following the commercial protocol. For intracytokine staining of IL-17, IL-4, and IFN-γ, splenocytes were treated for RBC lysis. Then, they were cultured at 5 million cells/ml in lymphocyte media with 50ng/ml PMA, 1μg/ml ionomycin, and 0.66μl/ml monesin for 6 hours at 37°C in a single well of a 24 well

culture dish. After incubation, the cells were centrifuged at 450xg for 5 minutes and stained using the BD Pharmigen Mouse Th1/Th2/Th17 Phenotyping Kit and its protocol.

Cell Sorting

Prior to the ELISPOT assay, splenocytes were sorted at the Children's Research Institute. Splenocytes were treated for RBC lysis and surface stained for MHCI (H-2kd) as described above. The cells were then sorted based on their MHCI (H-2kd) expression into 15ml tubes containing 3ml of lymphocyte media.

AUTOANTIBODY QUANTIFICATION

Sample Preparation

100µl of blood was collected via submandibular bleeding into a tube with EDTA. Samples were centrifuged at 450xg for 8 minutes and plasma was removed. Plasma was maintained at -80°C for long term storage.

IgG anti-dsDNA ELISA

The Mouse anti-dsDNA IgG-specific ELISA Kit from Alpha Diagnostic International was utilized to quantitate the activity of IgG anti-dsDNA antibodies in the plasma. Plasma was diluted following the commercial protocol and analyzed at 1/1000 and 1/10000. Additionally in every assay, an NZB/NZW F1, C57BL/6J, and blank sample were run as controls.

Autoantigen Microarray

The University of Texas Southwestern Medical Center Genomics and Microarray Core Facility completed the Autoantigen Microarray Panel I for IgG and IgM isotypes in the plasma samples [323]. Briefly, the plasma is separated into IgG and IgM isotypes, placed onto microarrays with known peptide autoantigens, and the binding affinity is monitored. During the analysis, the samples are normalized based on total IgG or IgM concentration and heat maps are generated based on relative values for the entire dataset.

KIDNEY PATHOLOGY

Histology

Mice were euthanized via CO_2 asphyxiation or isofluorane overdose and subsequent cervical dislocation. Kidneys were collected and bisected longitudinally. For routine stains, they were fixed in 4% paraformaldehyde for 24 hours at 4°C with shaking. Afterward, the kidneys were washed and stored in PBS at the University of Southwestern Histo Pathology Core Facility where they were embedded in paraffin, sectioned, and stained for Periodic-Acid Schiff (PAS), trichrome, and H&E. For immunofluorescence staining, the kidneys were bisected longitudinally and immediately cryo-embedded in OCT at the University of Southwestern Histo Pathology Core Facility where they were further sectioned at 5μm and placed onto slides. The slides were stored at -80°C until analysis. To stain the slides, they were first thawed at room temperature and then placed into -20°C acetone for 5 minutes. The slides were then washed twice in PBS for 5 minutes each and edged with a PAP pen. To block, 0.05% BD Pharmigen CD16/CD32 FC Block in 3% FBS in PBS was added for 30 minutes at room temperature. The block solution was then removed and FITC-conjugated anti-mouse IgG (Southern Biotech 1030-02) or FITC-conjugated goat IgG $F(AB)_2$ anti-C3 (0855510 MP Biomedical) was added at 1/50 in 3% FBS in PBS for 1 hour at room temperature protected from light. The slides were then washed in PBS three times for 5 minutes each. A 300nM DAPI solution was added at room temperature for 5 minutes. Then, the slides were washed

three times with PBS. Afterward, mounting media was added on the slides and secured with a glass coverslip. These slides were then stored at 4°C protected from light. Upon analysis, slides were photographed using Zeiss Axio Imager M1 fluorescent microscope and Axiovision software. Images were then formatted using ImageJ.

TEM

Kidneys were harvested, minced with a razor, and brought to the University of Texas Southwestern Electron Microscopy Core Facility where they were embedded and sectioned. Sections were analyzed using a FEI Technai G^2 Spirit TEM microscope and FEI Xplore3D tomography acquisition software.

Pathology Scoring

Slides from NZB/NZW F1, NZW/LacJ, NZB/BINJ, NZB;NZW, and NZB$^{Cd3e-/-}$;NZW embryonic chimeras were double-blinded and scored by Dr. Jose Torrealba, M.D., a renal pathologist at the Clements University Hospital. The scoring criterion used to identify the activity and chronicity of kidney disease was based on "The classification of glomerulonephritis in systemic lupus erythematosus revisited" [305], using PAS, H&E, trichrome, immunofluorescence staining, and TEM images from each mouse.

Proteinuria Quantification

Urine was collected from the mice via palpation and expression of the bladder at a single time point. The samples were stored on ice and brought to the University of Texas Southwestern Metabolic Phenotyping Core Facility for analysis on the VITROS 250 Microslide for total protein and creatinine levels.

GENERATION OF EMBRYONIC CHIMERAS

Derivation of Embryonic Stem Cells

Harvesting Embryos

3-4 week old female mice were injected with 200μl of 25 IU/ml PMSG at 2pm. 47 hours later, the mice were injected with 200ul of 25 IU/ml hCG and mated with stud males. The following morning, the females were checked for plugs. Two days later, the embryos were harvested at the morula stage. To collect the embryos, mice were euthanized via CO_2 asphyxiation and subsequent cervical dislocation. The oviducts were removed from the uterus and ovaries and placed into warm KSOM or FHM. After all oviducts were collected, they were flushed with KSOM or FHM using a 1ml syringe and blunted needles.

Embryo Culture

ESCs were derived based on the protocol in "Derivation and characterization of mouse embryonic stem cells from permissive and nonpermissive strains" [56]. After harvesting, embryos were collected from the media using a pulled glass pipette and transferred into warmed droplets of M-16 media under oil. The embryos were incubated at 37°C, 5% CO_2 for approximately 48 hours until they begun to hatch or were fully hatched. At this point, the blastocysts were transferred individually into a 15.6 mm diameter well in a 4 well plate with pre-plated irradiated MEF and the appropriate culture media. It was then left to incubate untouched. After 3 days, half the media was replaced with fresh media, and this was repeated every other day until outgrowths were large and apparent. At day 7 -10 after plating, the embryos were picked and disaggregated in 0.05% Trypsin-EDTA. Outgrowths were broken into fragments using a 32G needle, and further enzymatically digested with pipetting to reduce the cells into a single-cell suspension. The cells were then plated into a 15.6 mm diameter well of a 24 well plate and maintained.

Embryonic Stem Cell Culture

NZB/BINJ ESC are preferably grown in serum-free 2i media but also KOMP media, while NZW/LacJ ESCs are preferably grown in serum-free 2i media but also ES media. When passaging ESCs, the wells were first washed with PBS and 0.05% Trypsin-EDTA was added to cover the bottom of the well. The wells were incubated for 5 minutes at 37°C and then

disaggregated using pipetting. A 1:1 volume of the appropriate media was added, and the cells were centrifuged at 150xg for 5 minutes. Afterward, the supernatant was removed, and the pellet was resuspended in the appropriate media. The cells were plated into a new well and incubated at 37°C, 5% CO_2. For confluent cultures, the new wells were plated at a 1/6 dilution. Before the addition of ESCs, plates were pre-plated with irradiated MEF in MEF media one day prior. The wells were then washed in PBS and replaced with the appropriate embryonic stem cell media after the MEF has attached to the well.

To freeze the cells, 800µl of the cell suspension after trypsinization was added with 100µl FBS and 100µl DMSO. The cells were placed in a cryovial and an ethanol freezing container at -80°C for 24 hours. Afterward, the cryovial was moved to liquid nitrogen for long-term storage.

Nucleofection

ESCs at early passages (<5) were transfected with CRISPR-containing DNA plasmids conferring puromycin resistance using the Lonza Mouse ES Cell Nucleofector kit following the commercial protocol. ECSs were cultured in 10cm dishes, where each 10cm dish was used per nucleofection. After nucleofection on the A-023 setting, the cells were incubated in 6 34.8 mm wells of a 6 well plate on puromycin-resistant irradiated MEF. After 24 hours, the cells were treated with 1.5µg/ml puromycin until the colonies were large. The colonies were

then isolated and disaggregated similarly to outgrowth disaggregation, and cultured in a 96 well plate containing puromycin-resistant irradiated MEF or gelatin-coated wells. Cells growing in the gelatin-coated wells were then genotyped by Dr. Xiaoming Zhan while the cells growing with the MEF were used for further culture. The plasmids and CRISPR targets were designed by Dr. Xiaoming Zhan and ligated into the pX459 v2.0 plasmid. The CRISPR target sequence for *Cd3e* was TCAGAAGCATGATAAGCACC.

Blastocyst Microinjection

Cell Preparation

Embryos were harvested as described above and cultured for 1-2 days until they reached a fully expanded blastocyst stage. Dr. Xiaohong Li and I performed the injections. First, the cells used for the microinjection were disaggregated in 0.05% Trypsin-EDTA for 5 minutes at 37°C. Then, a 1:1 ratio of the appropriate media was added and the cells were centrifuged at 150xg for 5 minutes. The supernatant was removed, the cells were resuspended in the appropriate media, and they were plated back into the original well for 25 minutes at 37°C to remove the irradiated MEF. The cells were then removed from the well and centrifuged at 150xg for 5 minutes. The supernatant was removed and the cells were resuspended in FHM for injection.

Blastocyst Injection

A microinjection dish was prepared with a drop of FHM containing the expanded blastocysts and a drop of the cell suspension under oil. The dish was placed on the microinjection stage and the injection needle was pre-filled with oil and then media, for enhanced control. The holding pipette was pre-filled with media, as well. The injection needle was filled with approximately 10-15 ES cells in media. Then, individually, the blastocysts were positioned on the holding pipette so that the blastocoel was available to the injection needle and the inner cell mass was protected. With a swift movement, the injection needle was inserted between two trophoblast cells and the ES cells were injected into the blastocoel. The injection needle was then slowly removed to prevent ESCs from spilling out, and the blastocysts then contracted. The embryos were then transferred to the culture dish for incubation until transfer into the uterus.

Embryo Transfer

Pseudopregnant CD1 females were used as the recipients for the embryo transfers. 3 Days prior to the transfer, CD1 females were mated with vasectomized CD1 stud males and they were checked for plugs the following morning. On the same day as blastocyst injection, the plugged CD1 females were prepared for embryo transfers. Each mouse was given ketamine/xylazene via intraperitoneal injection and allowed to reach unconsciousness. Afterward, buphrenorphrine and carprofen were given via intraperitoneal injection and the

surgical region was shaved and disinfected following IACUC guidelines. During the surgery, the mouse was placed under a dissection microscope on a heating pad to maintain body temperature. Using sterile surgical techniques, an incision into the abdominal cavity was created and the oviduct was exposed. A small hole was made into the proximal uterus using a needle, and the embryos were transferred into the uterus using a pulled pipette. The abdominal wall was then sutured closed with polyglactin sutures, and the skin was closed with staples. The mouse was then given atipamezole and allowed to wake up on a heating pad. Once the mouse was upright and mobile, she was placed back her cage and allowed to gestate and give birth naturally.

Evaluation of Chimerism

Pups born from the transferred embryos were first separated based on visible skin chimerism. Blood was collected via submandibular collection from mice with any visible chimerism and analyzed on FACS for the percentage of NZB and NZW present among the lymphocytes, as described previously. Mice with between < 1% of either strain present in the PB were considered to be non-hematopoietic chimeras. Mice with between 1% and 10% were excluded from further analysis, as the cell number of one strain may be too low to identify the specific cell interactions being investigated in this study. Mice with between 10% to 90% present of either strain were used for further analysis. Out of 78 mice tested, 9 were excluded.

IN VITRO CELL ASSAYS

ELISPOT

One day prior to sorting the cells, the ELISPOT plate (Milipore MAIPS4510) was activated by adding 15ul of 35% ethanol to each well. Immediately after adding, the wells were washed twice with water and coated with 20μg/ml of poly-L-lysine. The plate was incubated at 37°C for 2 hours. The plate was then washed with 0.05% Tween-20 in PBS five times, incubating the wells with each wash for 1 minute. Then, the wells were coated with 20mg/ml calf thymus DNA at 4°C overnight.

The next day, the plate was washed five times with 0.05% Tween-20 in PBS, blocked with 10% FBS in PBS for 1 hour at 37°C, and again washed five times with 0.05% Tween-20 in PBS. Splenocytes were sorted from mice as described previously based on MHCI haplotype, and $5x10^5$ cells were added to each well for 2 hours at 37°C. Afterward, the cells were lysed in water for 1 minute twice, and then washed five times. The plate was incubated with biotinylated anti-mouse IgG (Jackson ImmunoResearch 115-065-008) at 1/1000 in 10% FBS in PBS at room temperature, and washed five times with 0.05% Tween-20 in PBS. To amplify the signal, BD ELISPOT streptavidin-HRP conjugate was added at 1/100 for 1 hour at room temperature, and washed 4 times with 0.05% Tween-20 in PBS and 3 times with PBS. The HRP substrate was prepared by dissolving 20mg of 3-amino-9-ethylcarbazole in

2ml of N,N-dimethylformamide. 333.33µl of this solution was added to 10ml of BD ELISPOT Acetate solution and filtered through a 0.45µM filter to remove undissolved precipitates. Immediately before addition to the plate 5µl of hydrogen peroxide was then added to the substrate solution. Finally, the substrate was added to each well for 5-10 minutes and spot development was monitored. To stop the reaction, water was added and used to wash the wells three times. Afterward, the water was removed and the plate was dried at room temperature overnight.

ELISPOT plates were read using a Bioreader instrument and software at the University of Texas Southwestern Immunology Department.

Mixed Lymphocyte Reaction (MLR)

Mice were euthanized via CO_2 asphyxiation or isofluorane overdose and subsequent cervical dislocation. Tissues were collected and stored in 3% FBS in PBS on ice until they were then disaggregated into a single cell suspension through a 40µm cell strainer in 6ml of 3% FBS in PBS using a syringe plunger. Samples were then centrifuged at 450xg for 5 minutes and the supernatant was removed via vacuum. Splenocytes were treated with 1.5ml RBC lysis buffer (SIGMA R7757) for 5 minutes at room temperature. It was then diluted in 9 ml of 3% FBS in PBS, centrifuged at 450xg for 5 minutes, and the supernatant was removed. Cells were washed in 10ml of PBS, and centrifuged at 450xg for 5 minutes to remove any excess protein. Cells were then diluted to $2x10^6$ cells/ml in 5% FBS in PBS. Stimulator NZB/BINJ,

NZW/LacJ, and C57BL/6J LN and splenocytes were stained with CellTrace FarRed Proliferation Dye. Stock solution of the FarRed Proliferation Dye (1mM) was diluted in PBS to 1μM. Responder NZB;NZW chimeras, NZB/BINJ, and NZW/LacJ were unstained. For the stimulators, 110μl of the diluted dye was added per ml of cell suspension, mixed thoroughly, and incubated at room temperature for 8 minutes, protected from light. Afterward, R5 media was added up to 40ml total volume, and the cells were centrifuged at 450xg for 5 minutes. The supernatant was removed and the stimulators were resuspended at $1x10^7$ cells/ml and the responders were resuspended at $2.5x10^6$ cells/ml in lymphocyte media. 100μl of the appropriate stimulators and responders were combined into round-bottom wells of a 96 well plates and incubated at 37°C and 5% CO_2. After 3 days, the cells were collected and analyzed via FACS using the BD Pharmigen Mouse Foxp3 fixation and permeabilization buffers and protocol using the Ki-67 proliferation panel.

CHAPTER FOUR
Results

THE AUTOIMMUNE PHENOTYPE OF NZB;NZW CHIMERAS

Overview

While the disease in the NZB/NZW F1 mice has been characterized for decades, utilizing the same strains to make NZB;NZW embryonic chimeras would not necessarily result in the same disease characteristics. The strains are interacting intercellularly in the chimera, whereas in NZB/NZW F1 they are interacting through epistasis. As a result, understanding the differences between NZB/NZW F1 and NZB;NZW chimeras provides insight into the importance of different genetic and cellular interactions in disease features.

The main features of autoimmunity seen in humans, such as those in the SLE diagnostic criteria, are not all seen in mice, or even in all individuals with SLE. Typically in mice features such as rashes, autoantibody production (dsDNA, ANA), renal disease, and lymphoid tissue pathology can be seen, and thus these were tested for in the NZB;NZW embryonic chimeras. Based on the characteristic disease progression seen in the NZB/NZW F1 mice that serve as the basis for the NZB;NZW mice, these features were tested monthly until 8 months of age, when NZB/NZW F1 female mice frequently succumb to disease. Similar to the NZB/NZW F1, the NZB;NZW embryonic chimeras do not develop any skin lesions upon visual inspection, and the other features were investigated experimentally.

Autoantibody Production

Anti-dsDNA Autoantibodies

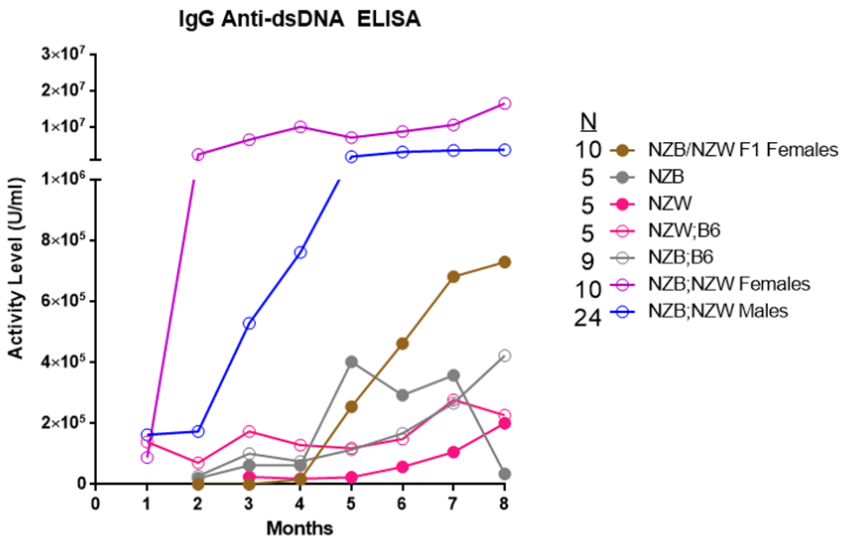
The production of IgG anti-dsDNA autoantibodies is the most specific and sensitive for SLE compared to other autoimmune diseases. A standard measure of "Activity Level" was utilized to reduce inter-assay variability. NZB/NZW F1 developed these autoantibodies as expected, beginning around 5 months of age and reaching maximum at 8 months. NZW females did not show appreciable titers of these autoantibodies, however NZB showed an initial increase at 5 months that eventually decreased and never reached levels comparable to the NZB/NZW F1. Strikingly, NZB;NZW chimeric females produced elevated levels of autoantibody as early as 1-3 months of age that reached titers more than 10-fold greater than those observed in NZB/NZW F1 females. NZB;NZW chimeric males also developed autoantibodies at approximately 3 months of age. No NZW;C57BL/6J chimeras and 11% (1/9) of the NZB;C57BL/6J chimeras produced anti-dsDNA antibodies. Some NZB;NZW chimeras showed skin chimerism but only had detectable NZW derived hematopoietic cells (NZB;NZW non-hematopoietic chimeras). 22% (10/45) of the NZB;NZW non-hematopoietic chimeras developed anti-dsDNA antibodies. Unlike the NZB;NZW chimeras, the NZB;NZW non-hematopoietic and NZB;C57BL/6J chimeras that produced autoantibodies did so at approximately 6 months of age (Figure 1A, B). This indicates that NZB hematopoietic cells promote autoimmunity, as the phenotype is fully penetrant, has an earlier onset, and stronger severity in hematopoietic chimeras than non-hematopoietic chimeras. Furthermore, the

poorly penetrant autoimmunity in the NZB;C57BL/6J and NZW;C57BL/6J chimeras demonstrates that generating chimeras with mismatched MHC does not inherently cause disease.

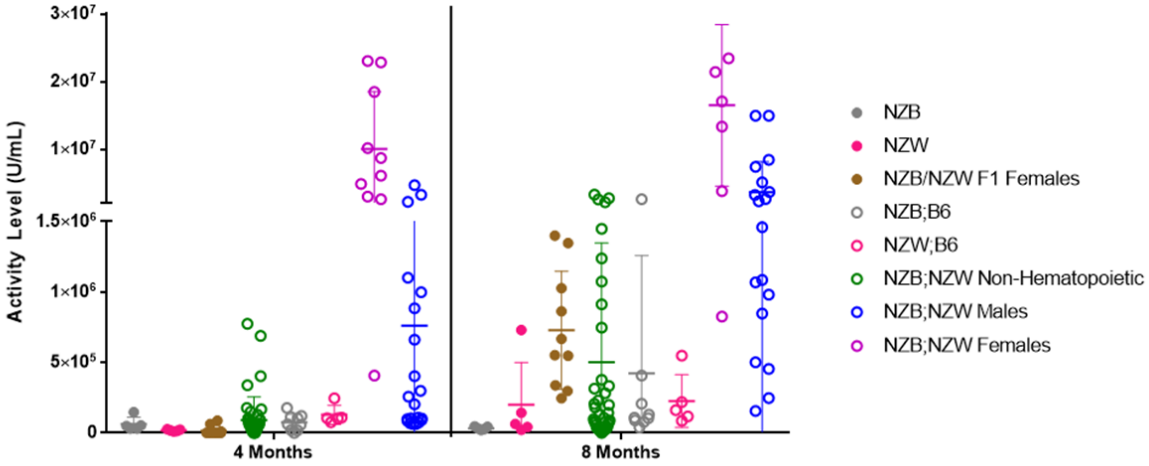
IgM and IgG Autoantibody Specificities

In SLE, individuals typically develop autoantibodies reactive with a diverse array of nuclear and self-antigens. To identify autoreactivity to these SLE-associated antigens, the NZB;NZW chimeric females' plasma IgG and IgM antibodies were tested using a protein microarray. Starting at 1-2 months, the NZB:NZW chimeric females' IgG and IgM showed reactivity with many self-antigens (Appendix A), including those associated with SLE (Figure 1C). As expected, NZB/NZW F1 hybrids show a similar pattern of autoreactivity at 8 months, whereas the NZW and NZB parental strains show very limited autoreactivity for IgG isotypes. NZB mice did show autoreactivity to many antigens, including nuclear antigens, for IgM isotypes.

A


IgG Anti-dsDNA ELISA
Activity Level (U/ml)
3×10^7
2×10^7
1×10^7
1×10^6
8×10^5
6×10^5
4×10^5
2×10^5
0
0 1 2 3 4 5 6 7 8
Months
N
10 NZB/NZW F1 Females
5 NZB
5 NZW
5 NZW;B6
9 NZB;B6
10 NZB;NZW Females
24 NZB;NZW Males

B


Activity Level (U/mL)
3×10^7
2×10^7
1×10^7
1.5×10^6
1×10^6
5×10^5
0
4 Months
8 Months
NZB
NZW
NZB/NZW F1 Females
NZB;B6
NZW;B6
NZB;NZW Non-Hematopoietic
NZB;NZW Males
NZB;NZW Females

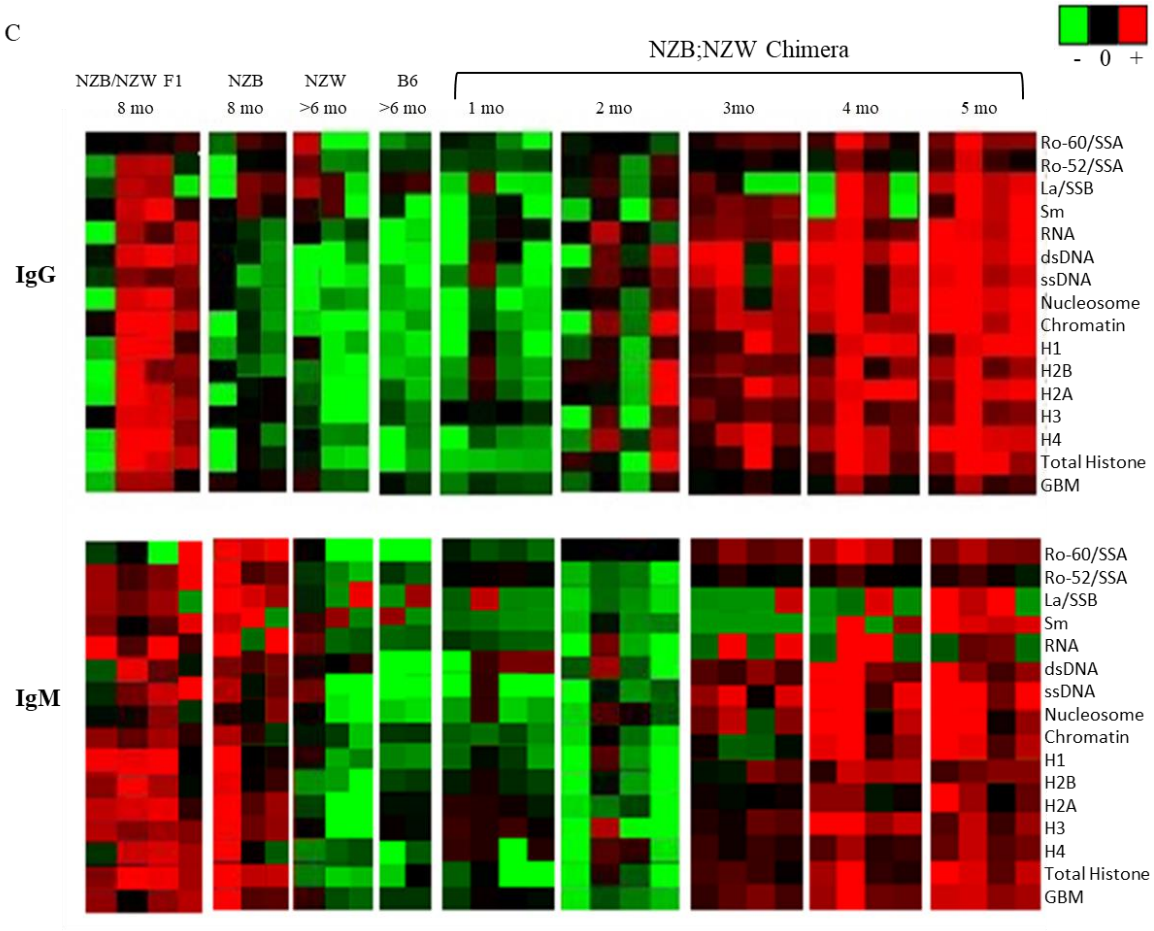


Figure 1. IgG anti-dsDNA Production in Mouse Models.

(A) Averaged and (B) individual values for the activity level of IgG anti-dsDNA antibodies in different mouse strains over time. (C) Plasma from mice was collected and analyzed on a peptide microarray for IgM and IgG isotypes. For the heatmap, values greater than the median are red, values at the median are black, and values below the median are green. GBM; glomular basement membrane.

Kidney Pathology

Urinalysis

In both mouse models and humans with SLE, the final stages of the disease can include glomerulonephritis. Deposition of immune complexes and direct binding of autoantibodies can lead to inflammation in the kidney and is the main cause of mortality in the NZB/NZW F1 mice and humans[24, 286]. In order to determine if this also occurs in the NZB;NZW chimeras we tested for elevated protein/creatinine ratios in the urine (Figure 2A). In NZB/NZW F1 the average protein/creatinine ratio at 9 months of age was 123.7mg/mg with a range of 6.9-343.7mg/mg. NZW mice at $\geq$9 months of age had an average of 0.89mg/mg with a range of 0.1-2.8mg/mg. And, NZB mice at $\geq$9 months of age had an average of 0.7mg/mg with a range of 0.4-1.2mg/mg. The NZB;NZW chimeras did have elevated protein/creatinine levels in the urine, however it was only significantly different than the NZW controls at 3-6 months of age and never consistently reached the levels seen in the NZB/NZW F1. While some of the NZB;NZW chimeras did show high concentrations of protein, it was not fully penetrant and not sex-dependent.

Histology

The kidneys of 9 month old NZB;NZW chimeric mice, NZB, NZW, and NZB/NZW F1 were analyzed for glomerulonephritis. They were given an activity/chronicity score and pathology score based on rigorous guidelines of pathogenic features[41, 305] using TEM,

immunofluorescence for IgG and C3 immune deposits, and H&E, trichrome, and periodic-acid shift stains (PAS) (Table 6) (Figure 2B, C). NZB mice consistently had little to no kidney pathology, with an activity/chronicity score of 0.6. NZW mice showed endocapillary hypercellularity and IgG immune deposits, resulting in an increased activity/chronicity score designation of 2.6. NZB/NZW F1 had severe glomuleronephritis, with an activity/chronicity score of 13. Meanwhile, NZB;NZW chimeric females had moderate endocapilliary hypercellularity, subendothelial hyaline deposits, interstitial inflammation, and IgG and C3 immune deposits, leading to an activity/chronicity score of 6.2 . However, this kidney disease was not fully penetrant and NZB;NZW chimeric males more closely resembled the wildtype NZW than NZB/NZW F1, with an activity/chronicity score of 4.8.

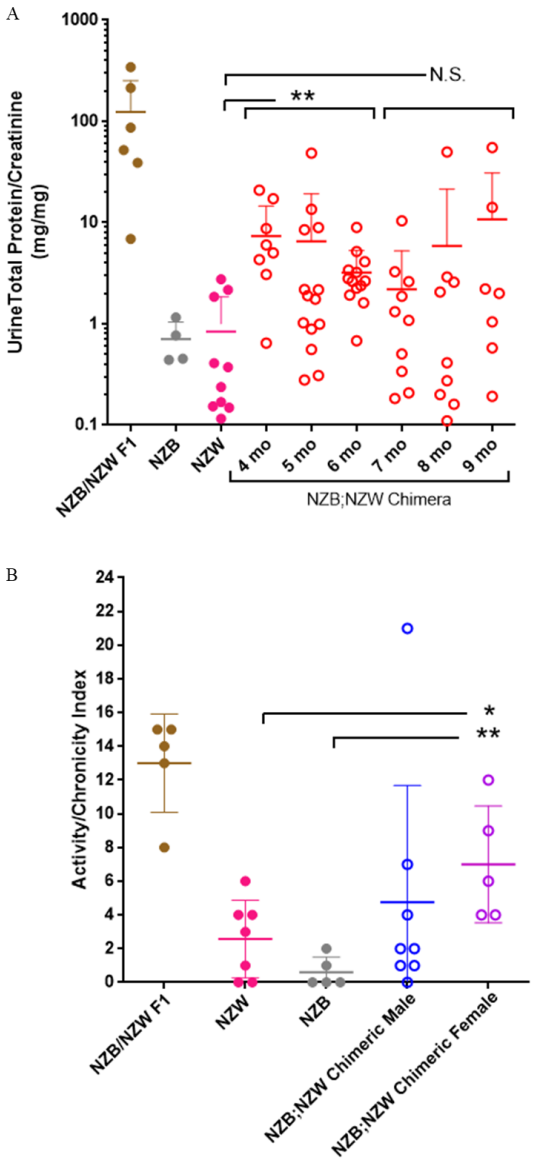


Figure 2. NZB;NZW Chimeras Develop Moderate Nephritis

(A) Urine was collected monthly and tested for total protein and creatinine. NZB/NZW F1, NZB/BINJ, and NZW/LacJ mice are ≥9 months of age. (B) Activity and Chronicity Index of each mouse in a blinded manner. (C) Representative images of various stains and TEM images from the kidneys collected from the mice. * = $p \leq 0.05$, ** = $p \leq 0.01$ using the Mann-Whitney test.

C

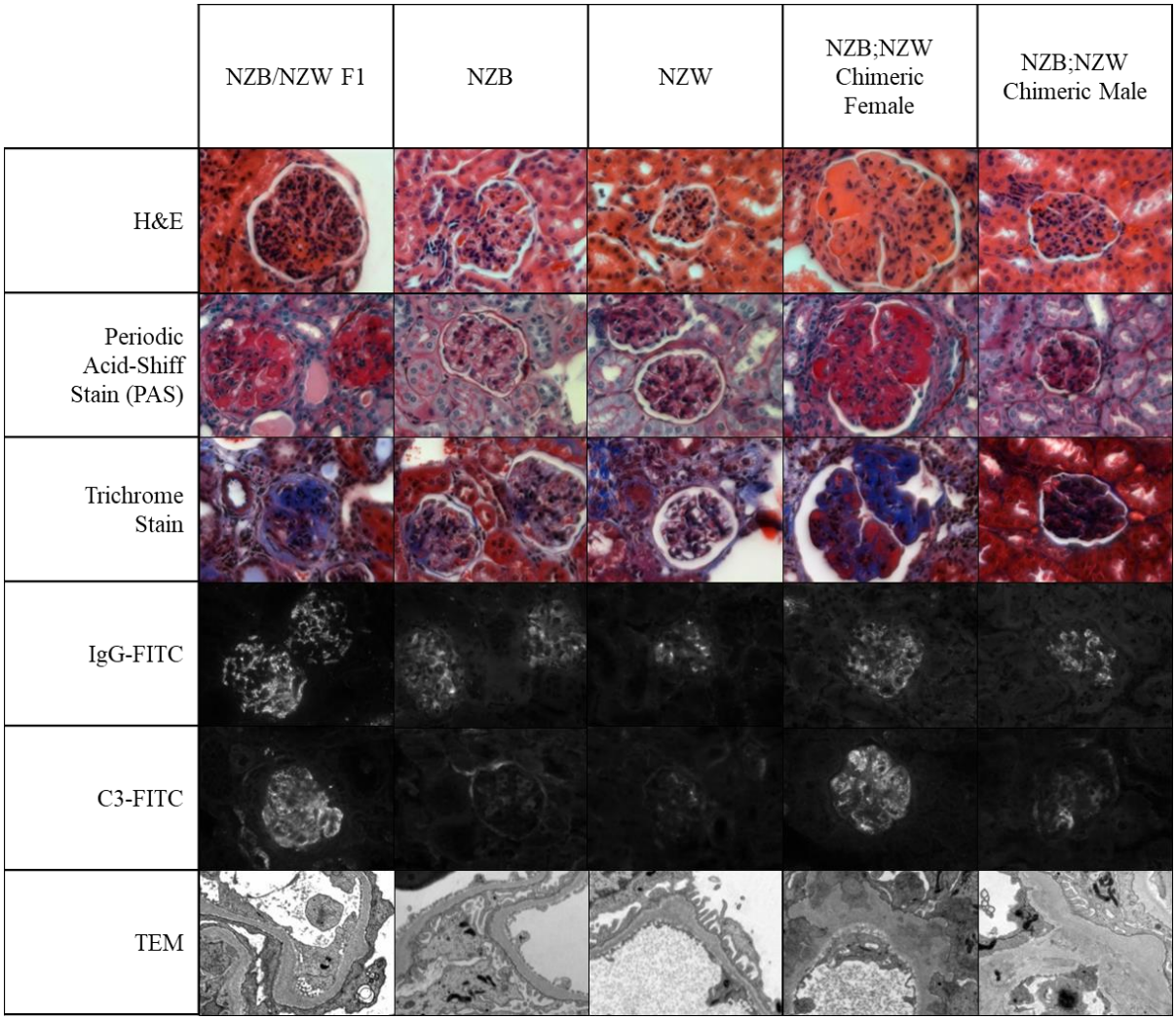

NZB/NZW F1
NZB
NZW
NZB;NZW Chimeric Female
NZB;NZW Chimeric Male
H&E
Periodic Acid-Shiff Stain (PAS)
Trichrome Stain
IgG-FITC
C3-FITC
TEM

Table 6: Activity and Chronicity Indexes of Renal Pathology and Nephritis Class in NZB;NZW Chimeras, NZB$^{Cd3e-/-}$;NZW Chimeras, and Controls

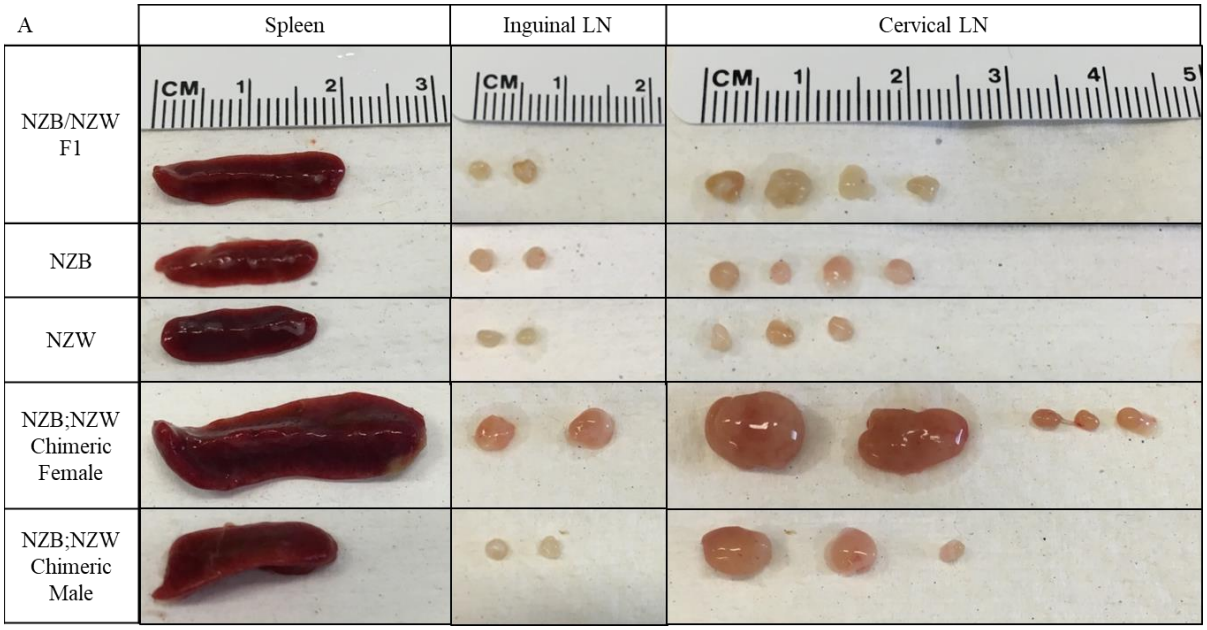
Activity Index		**NZW**	**NZB**	**NZB/NZW F1**	**NZB;NZW Females**	**NZB;NZW Males**	**NZB$^{Cd3e-/-}$;NZW**
Feature	**Range**						
Endocapillary hypercelluarity	0-3+	1.6	0.4	3.0	2.6	1.6	0.9
Subendothelial hyaline deposits	0-3+	0.4	0.0	2.6	2.0	1.6	0.4
Fibrinoid necrosis/ karyorrhexis	0-3+ (x2)	0.0	0.0	0.2	0.0	0.1	0.0
Cellular crescents	0-3+ (x2)	0.0	0.0	0.2	0.0	0.2	0.0
Interstitial inflammation	0-3+	0.4	0.0	2.2	1.2	1.1	0.1
TOTAL SCORE	0-21	2.4	0.4	8.6	5.8	4.8	1.4
Chronicity Index		**NZW**	**NZB**	**NZB/NZW F1**	**NZB;NZW Females**	**NZB;NZW Males**	**NZB$^{Cd3e-/-}$;NZW**
Feature	**Range**						
Glomerular sclerosis	0-3+	0.0	0.2	1.4	0.4	0.5	0.0
Fibrous crescents	0-3+	0.0	0.0	0.2	0.0	0.1	0.0
Tubular atrophy	0-3+	0.0	0.0	1.4	0.4	0.3	0.1
Interstitial fibrosis	0-3+	0.1	0.0	1.4	0.4	0.3	0.1
TOTAL SCORE	0-12	0.1	0.2	4.4	1.2	1.1	0.3
Activity + Chronicity	**0-33**	**2.6**	**0.6**	**13.0**	**6.2**	**4.8**	**1.6**
Nephritis Class	**0-5**	**2.7**	**0.8**	**4.0**	**4.0**	**2.3**	**2.0**

Lymphoid Organ Pathology

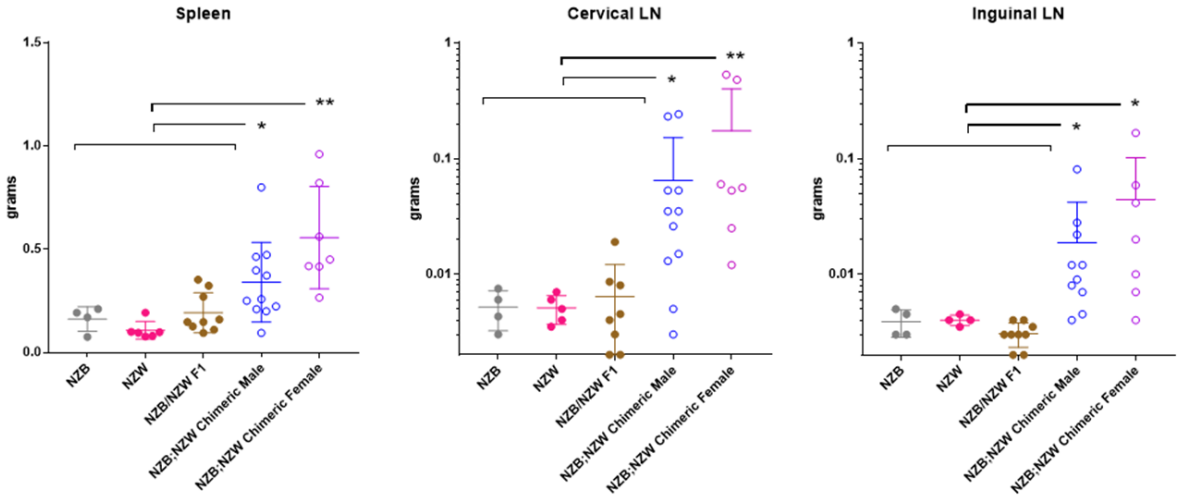
Another feature seen in SLE is enlarged lymphoid organs, such as the LN and spleen. This is typically seen in mouse models of SLE, and occasionally in SLE patients. NZB and NZW mice do not typically develop lymphadenopathy or splenomegaly, while NZB/NZW F1 develop these features. The NZB;NZW chimeras develop enlarged cervical and inguinal lymph nodes that can be visualized and palpated externally by 5 months of age (Figure 3A). Furthermore, the lymph nodes for male and female NZB;NZW chimeras weighed significantly greater than NZB/NZW F1, NZB, or NZW upon isolation at 8-9 months of age (Figure 3B). Although the severe lymphadenopathy is distinct from the NZB/NZW F1, similar LN are seen in the MRL/*lpr* strain. H&E slides were prepared and analyzed by Dr. Bret Evers. This enlargement is due to the expansion of the paracortex with polymorphic lymphocytes and high-endothelial venules in chimeric animals compared to those of controls, which contain relatively smaller paracortical areas and fewer associated components (Figure 3C). Scattered follicles are present in both groups.

The spleens of female but not male NZB;NZW chimeras weighed significantly greater than NZB/NZW F1, NZB, or NZW (Figure 3B). The splenomegaly is attributed to expansion of the white pulp in chimeric animals compared to controls (Figure 3D). Additionally, all groups show extramedullary hematopoiesis to variable extents.

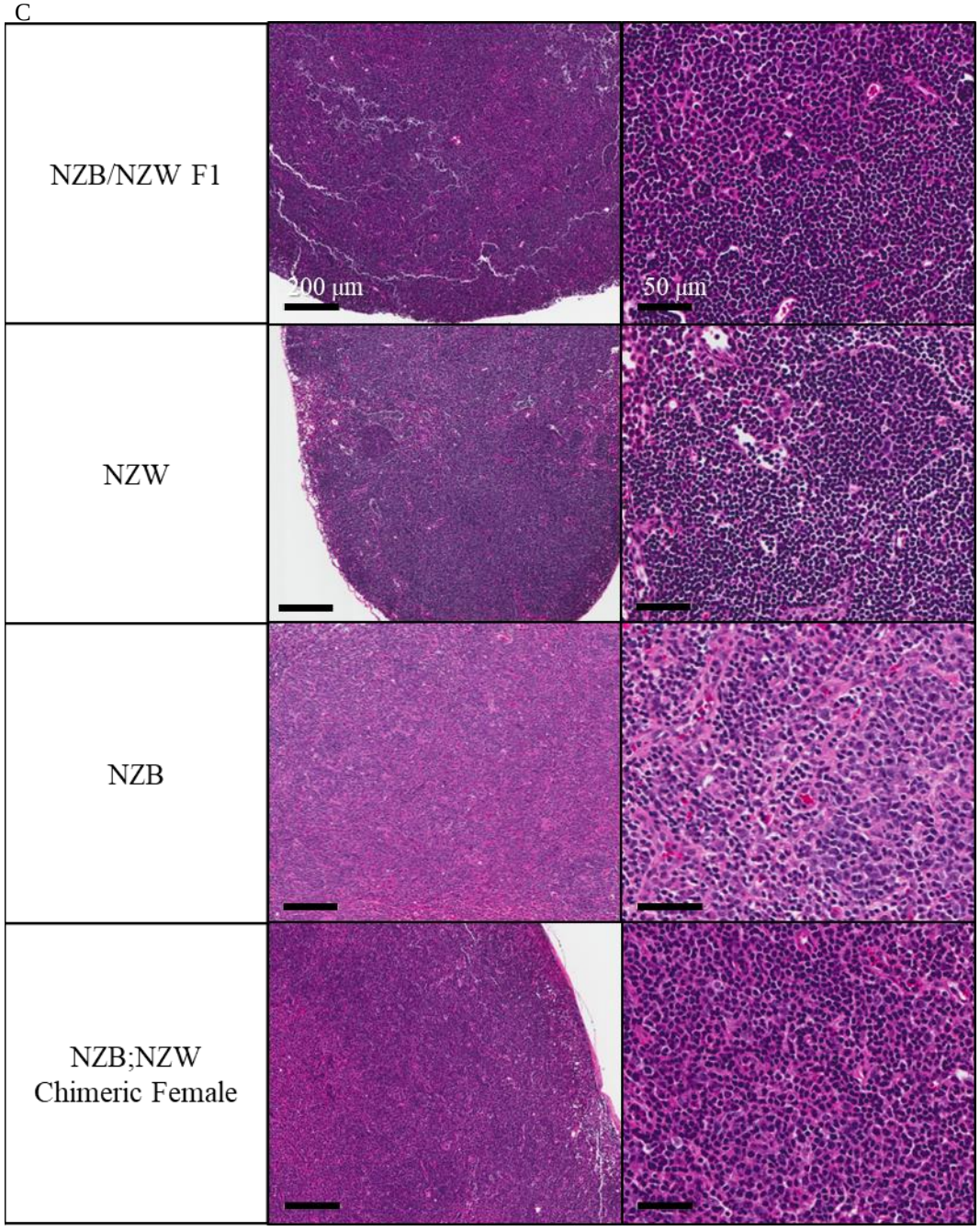
A


Spleen
Inguinal LN
Cervical LN
NZB/NZW F1
NZB
NZW
NZB;NZW Chimeric Female
NZB;NZW Chimeric Male

B


Spleen
Cervical LN
Inguinal LN
grams
NZB
NZW
NZB/NZW F1
NZB;NZW Chimeric Male
NZB;NZW Chimeric Female

C



D

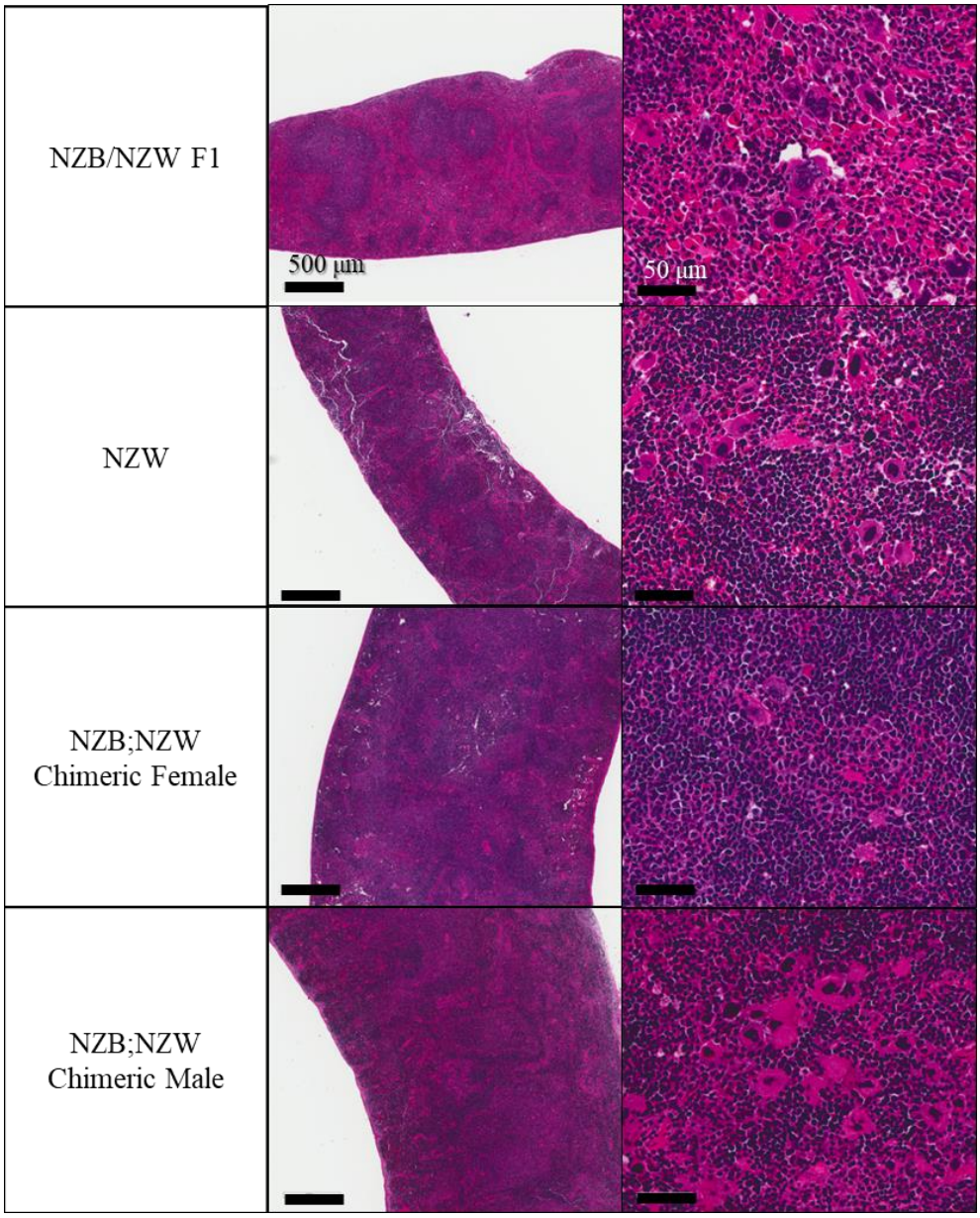


Figure 3. NZB;NZW Chimeras Develop Lymphadenopathy and Splenomegaly

(A) Spleens, inguinal LN, and cervical LN were collected from 9 month old mice and weighed (g) (B). (C) H&E stained cervical LN and (D) spleens from NZB;NZW chimera, NZW, NZB, and NZB;NZW F1 mice. * = $p \leq 0.05$, ** = $p \leq 0.01$ using the Mann-Whitney test.

Table 7: Characteristics of Mouse Strains at 6-8 months.

Mouse Strain	Lymphoid Organs	Kidney Pathology	Autoantibody
NZB/NZW F1	Splenomegaly. Average Spleen Weight = 193mg Lymphadenopathy. Average LN Weight = 6.4mg	Severe glomerulonephritis with immune deposits. Average Activity/Chronicity = 13 Average Nephritis Class = 4 Severe Proteinuria Average value = 123.7mg/mg.	Develops around 5 months and reaches highest titers at 8 months. Females have earlier onset than males. Broad autoreactivity in IgG and IgM isotypes.
NZW/LacJ	Normal. Average Spleen Weight = 108mg Average LN Weight = 5.2mg	Slight kidney pathology. Average Activity/Chronicity = 2.6 Average Nephritis Class = 2.7 Normal Urine Protein Average value = 0.89mg/mg	None detected.
NZB/BinJ	Normal. Average Spleen Weight = 163mg Average LN Weight = 5.2mg	No glomerulonephritis. Average Activity/Chronicity = 0.6 Average Nephritis Class = 0.8 Normal Urine Protein Average value = 0.70mg/mg	Low autoreactivity in IgG isotypes, broad autoreactivity in IgM isotypes.
NZB;B6 Chimera	Normal.	Not Tested.	11% (1/9) show anti-dsDNA IgG. Develops around 5 months.

Table 7: Characteristics of Mouse Strains at 8 months, continued.

Mouse Strain	Lymphoid Organs	Kidney Pathology	Autoantibody
NZW;B6 Chimera	Normal.	Not Tested.	None detected.
NZB;NZW Chimera (Male)	Splenomegaly. Average Spleen Weight = 319mg Lymphadenopathy Average LN Weight = 6.0mg	Slight glomerulonephritis. Average Activity/Chronicity = 4.8 Average Nephritis Class = 2.3 Urine Protein Above Normal. Average value = 1.09mg/mg.	Develops around 3 months and reaches levels 10 fold greater than F1. Broad autoreactivity in IgG and mainly chromatin and dsDNA autoreactivity in IgM isotypes.
NZB;NZW Chimera (Female)	Splenomegaly. Average Spleen Weight = 506mg Lymphadenopathy Average LN Weight = 153.8mg	Slight glomerulonephritis but more than NZB;NZW chimeric males. Average Activity/Chronicity = 6.2 Average Nephritis Class = 4 Urine Protein Above Normal. Average value = 24.9mg/mg.	Develops around 1-2 months and reaches levels 10 fold greater than F1. Broad autoreactivity in IgG and mainly chromatin and dsDNA autoreactivity in IgM isotypes.
NZB;NZW Chimera Non-hematopoietic	Normal.	Not Tested.	23% (10/44) show anti-dsDNA IgG. Develops around 6 months.
NZB$^{Cd3e-/-}$;NZW Chimera	Normal.	Normal. Average Activity/Chronicity = 1.6 Average Nephritis Class = 2 Urine Protein Normal. Average value = 0.56mg/mg.	14% (4/29) show anti-dsDNA IgG. Develops around 5 months.

CHAPTER FIVE
Results

CELLULAR PATHWAYS THAT PROMOTE AUTOIMMUNITY IN NZB;NZW CHIMERAS

Overview

Understanding what causes SLE and the pathways involved in the amplification of the symptoms is necessary to find targeted therapies. Currently, the main therapeutics for SLE include steroids and new biologics, such as belimumab. While steroids are non-specific and have considerable side-effects, it has been the golden standard for SLE treatment. On the other hand, newly designed biologics are targeted to prevent B cell maturation, but have limited efficacy.

While it is likely that the diagnosis of SLE includes many different etiologies and pathways that yield the same result, it is important to identify these different etiologies so individuals can be treated appropriately. Furthermore, it will decipher aberrant pathways to help understand fundamental tolerance mechanisms.

Although B cells have largely been the focus in SLE due to the production of pathogenic autoantibodies, increasing evidence has highlighted the role of T cells in the disease through direct cytotoxic and B helper functions. Through an unbiased approach in the NZB;NZW

chimeras, NZB T cells were found to greatly accelerate SLE development and was required for strong SLE-like disease.

Deviations in Immune Cell Subsets

In order to determine the relative proportions of NZB and NZW cells in the major immune cell subsets, the peripheral blood (PB), spleen, inguinal, and cervical LN were collected from the mice at 9 months of age and analyzed by flow cytometry. The cell populations were gated on the parental strains' class I MHC antigen and then further gated for different cell populations. If any major populations had low expression of MHCI they were excluded from NZB/NZW phenotyping analysis.

Peripheral Blood (PB), Lymph Nodes (LN), Spleen

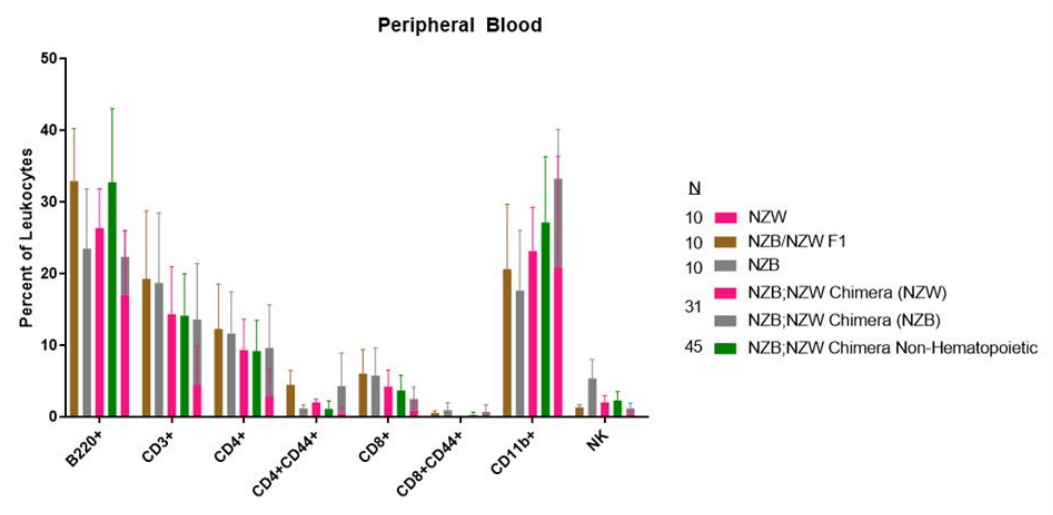
In all the organs studied, the major cell populations were represented in ratios similar to those observed in wildtype mice, indicating no major immune deficiency nor a remarkable expansion of any cellular compartment (Figure 4A, E, H, K). However, the development of the NZB and NZW cells into T and B cells was disproportionate. Moreover, a difference was observed upon comparison of NZB;NZW chimeras to NZB;C57BL/6J or NZW;C57BL/6J chimeras. In the PB and spleen of NZB;C57BL/6J chimeric mice, NZB cells predominately developed into B cells. On the other hand, in NZB;NZW chimeras, the majority of NZB cells were T cells (Figure 4 B, F, I, L). Due to this increase of T cells from the NZB lineage, when

considering the organ as a whole, the majority of the T cells are derived from NZB, while the majority of the B cells are from NZW (Figure 4 C, G, J, M). In the NZB;NZW chimeric females, the majority of CD4+ T cells, CD8+ T cells, and NK cells were derived from the NZB strain (~80%). An even greater proportion of CD44+ T cells were of NZB origin (~90%). On the other hand, most of the B cells were derived from the NZW strain (~70%). This phenotype does not develop progressively but is seen at the earliest time point tested in PB at 2 months and continued over time (Figure 4D). However, this unequal distribution was less apparent in NZB;NZW chimeric males, as the NZB parental strain had a lower frequency of T cells.

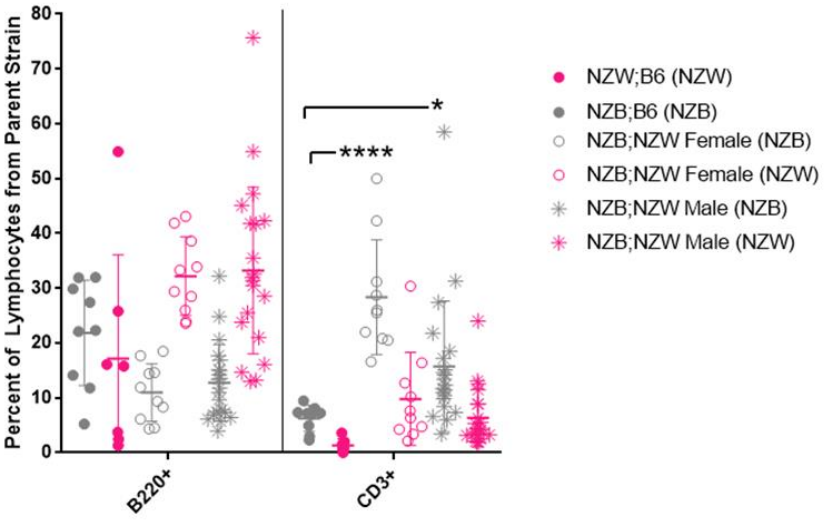
Figure 4. NZB;NZW Chimeras Have Disproportionate T and B Cell Contribution to Major Immune Cell Subsets.

(A, E, H, K) Averaged percent of cell populations in total leukocytes for wildtype mice and NZB;NZW Chimeras at 8-9 months of age. Stacked bars represent the combined total of NZB and NZW cells within the chimeras. (B, F, I, L) Individual value of the percent of T or B cells in the cells of the parental strain. (C, D, G, J, M) Individual values of the percent of each cell population that comes from NZB origin. * = $p \leq 0.05$, ** = $p \leq 0.01$, **** = $p \leq 0.0001$ using multiple T tests.

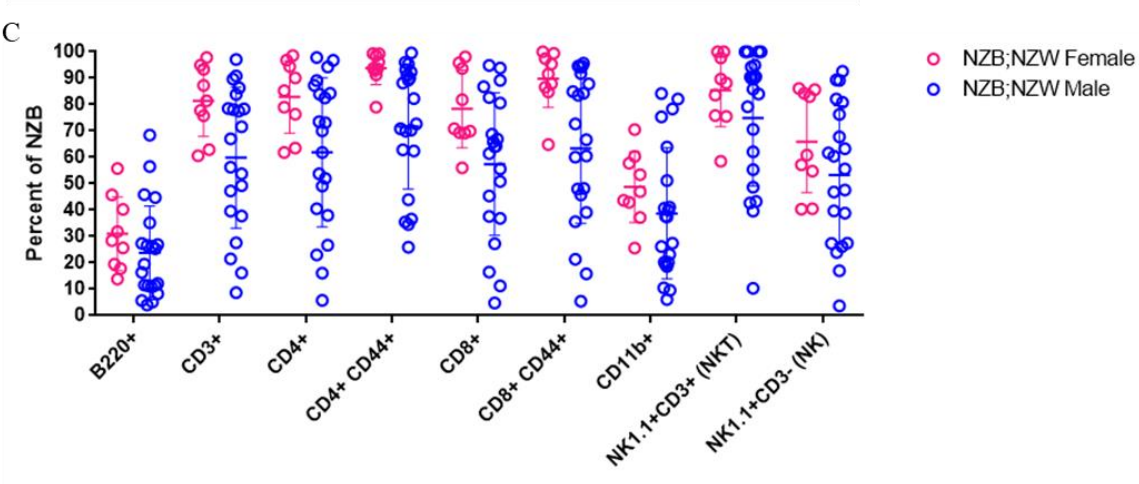
A


Peripheral Blood
Percent of Leukocytes
50
40
30
20
10
0
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+
NK
N
10 NZW
10 NZB/NZW F1
10 NZB
31 NZB;NZW Chimera (NZW)
NZB;NZW Chimera (NZB)
45 NZB;NZW Chimera Non-Hematopoietic

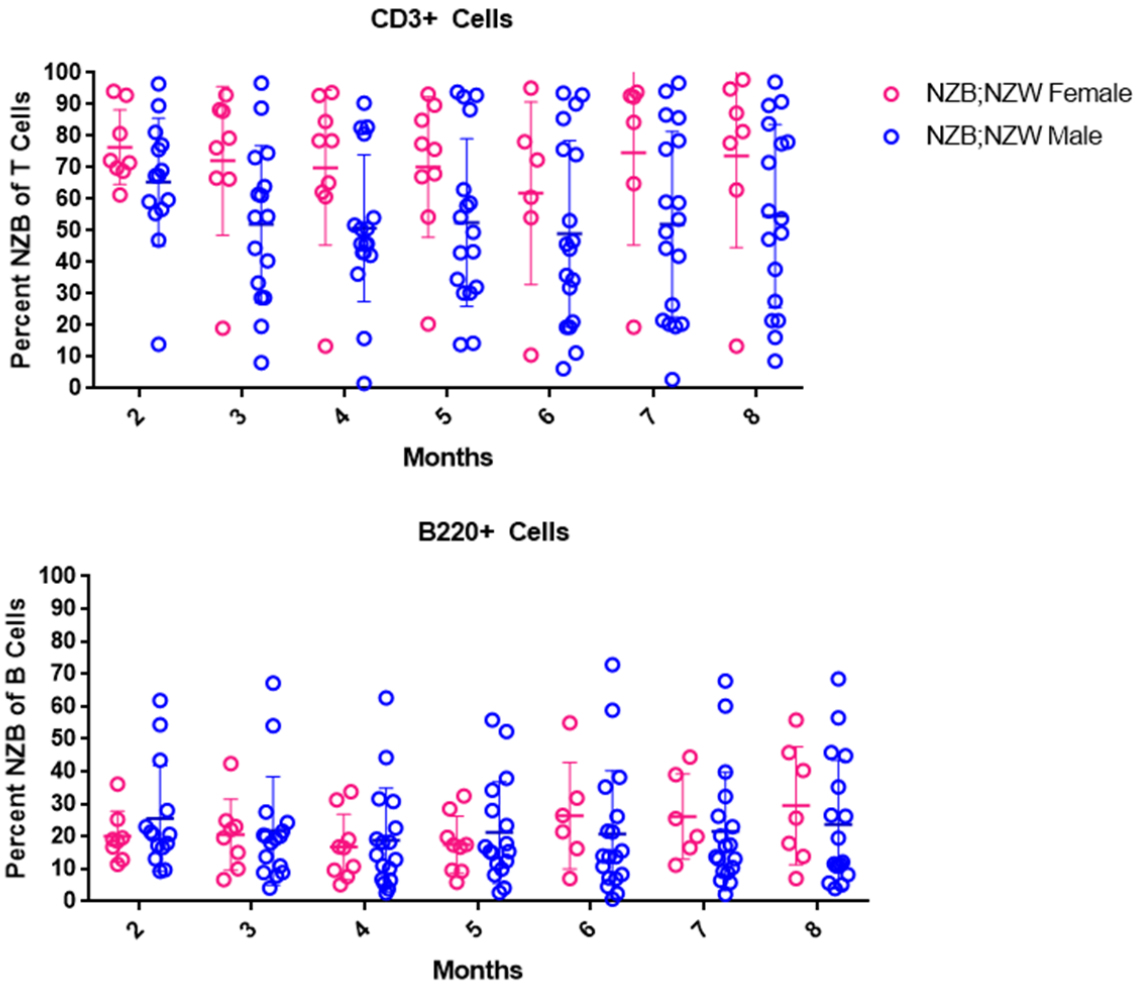
B


Percent of Lymphocytes from Parent Strain
80
70
60
50
40
30
20
10
0
B220+
CD3+
*
****
NZW;B6 (NZW)
NZB;B6 (NZB)
NZB;NZW Female (NZB)
NZB;NZW Female (NZW)
NZB;NZW Male (NZB)
NZB;NZW Male (NZW)

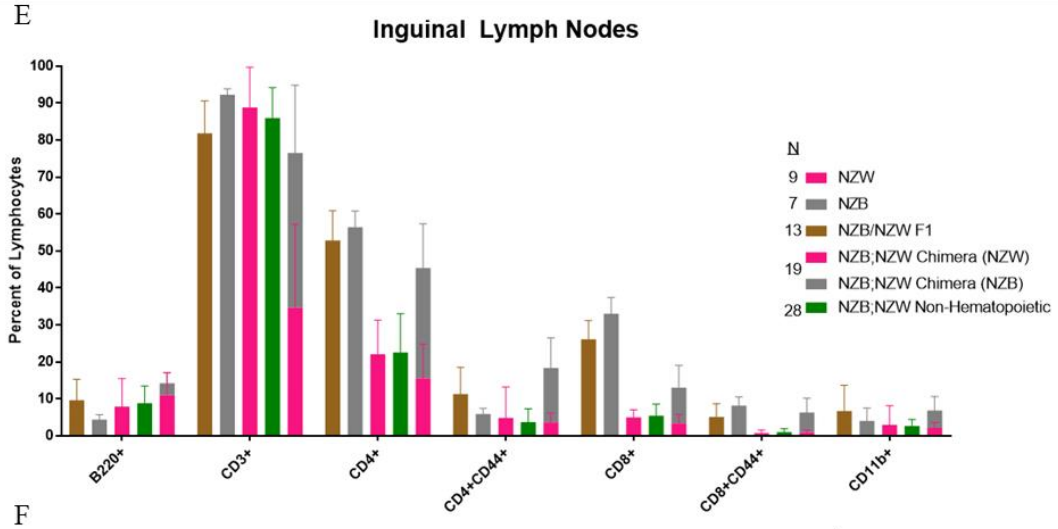
C


Percent of NZB
100
90
80
70
60
50
40
30
20
10
0
B220+
CD3+
CD4+
CD4+ CD44+
CD8+
CD8+ CD44+
CD11b+
NK1.1+CD3+ (NKT)
NK1.1+CD3- (NK)
NZB;NZW Female
NZB;NZW Male

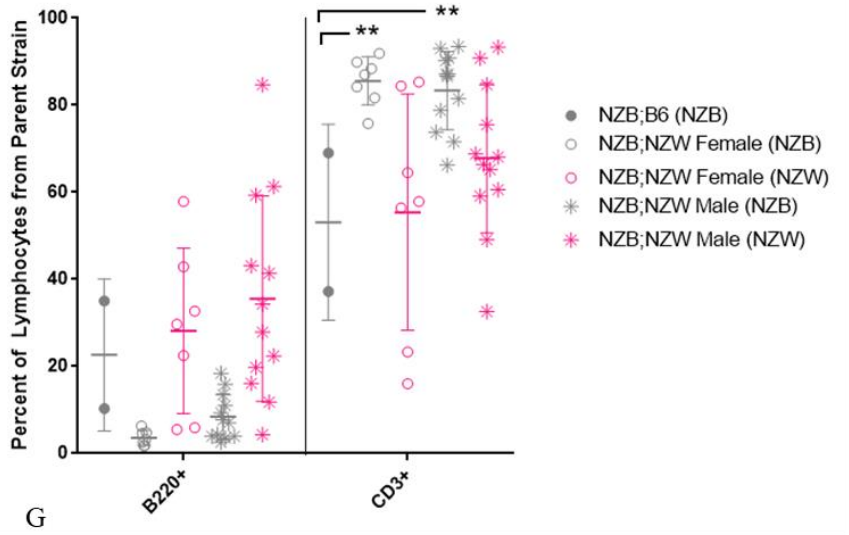
D


CD3+ Cells
Percent NZB of T Cells
100
90
80
70
60
50
40
30
20
10
0
2
3
4
5
6
7
8
Months
NZB;NZW Female
NZB;NZW Male
B220+ Cells
Percent NZB of B Cells
100
90
80
70
60
50
40
30
20
10
0
2
3
4
5
6
7
8
Months

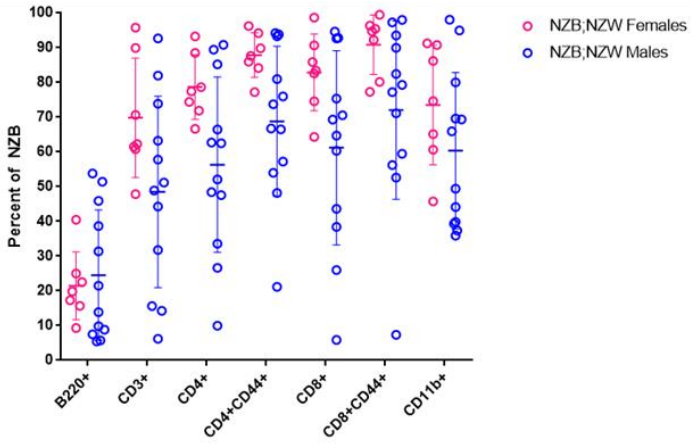
E


Inguinal Lymph Nodes
Percent of Lymphocytes
N
9 NZW
7 NZB
13 NZB/NZW F1
19 NZB;NZW Chimera (NZW)
NZB;NZW Chimera (NZB)
28 NZB;NZW Non-Hematopoietic
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+

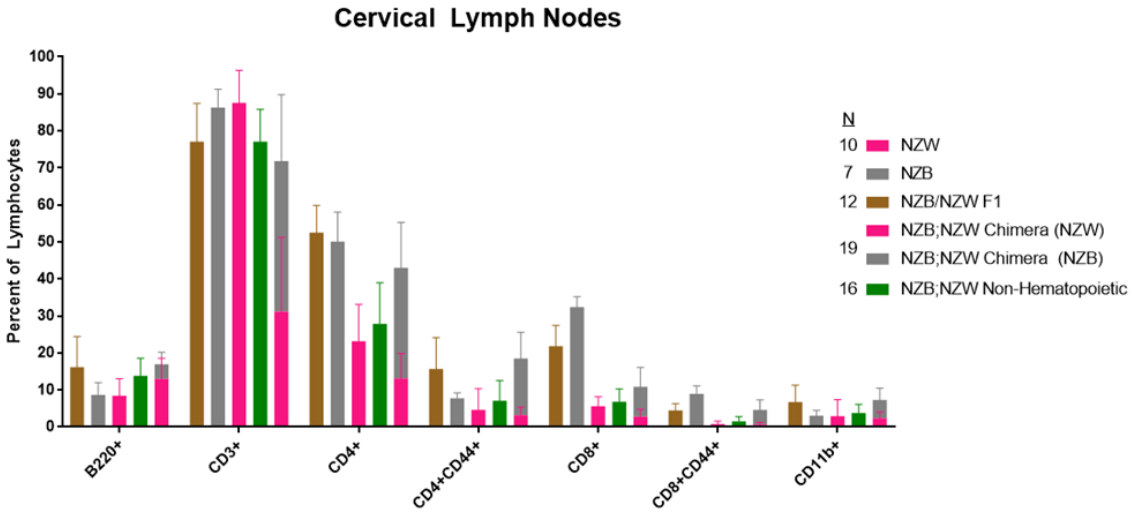
F


Percent of Lymphocytes from Parent Strain
**
**
NZB;B6 (NZB)
NZB;NZW Female (NZB)
NZB;NZW Female (NZW)
NZB;NZW Male (NZB)
NZB;NZW Male (NZW)
B220+
CD3+

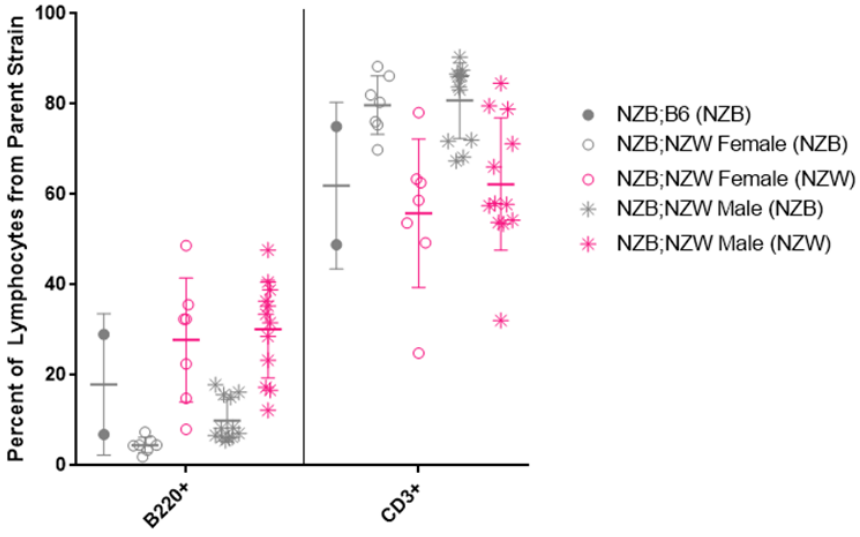
G


Percent of NZB
NZB;NZW Females
NZB;NZW Males
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+

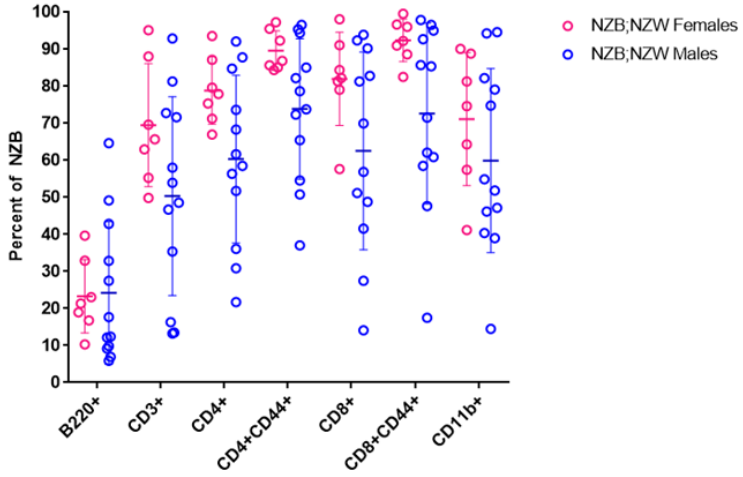
H


Cervical Lymph Nodes
Percent of Lymphocytes
100
90
80
70
60
50
40
30
20
10
0
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+
N
10 NZW
7 NZB
12 NZB/NZW F1
19 NZB;NZW Chimera (NZW)
NZB;NZW Chimera (NZB)
16 NZB;NZW Non-Hematopoietic

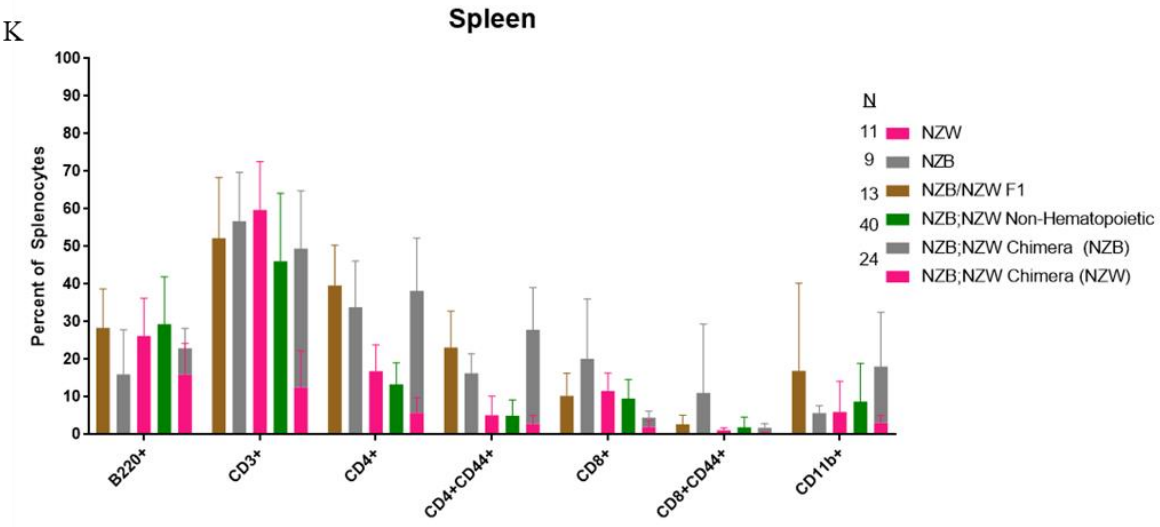
I


Percent of Lymphocytes from Parent Strain
100
80
60
40
20
0
B220+
CD3+
NZB;B6 (NZB)
NZB;NZW Female (NZB)
NZB;NZW Female (NZW)
NZB;NZW Male (NZB)
NZB;NZW Male (NZW)

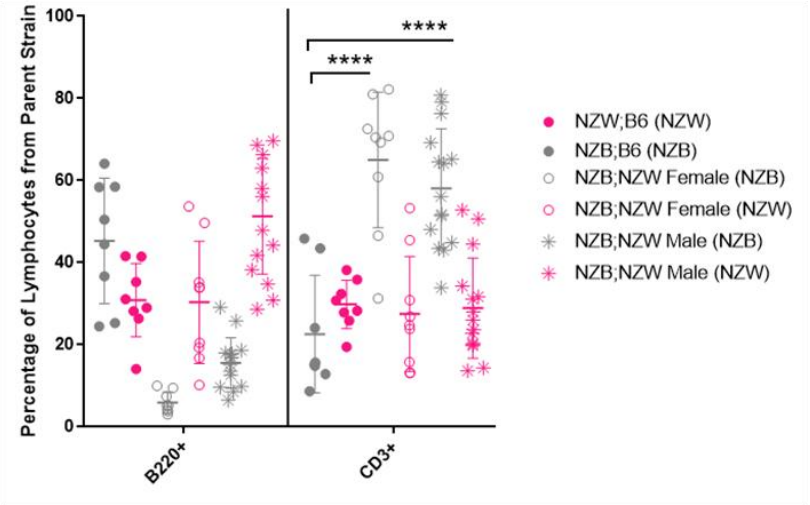
J


Percent of NZB
100
90
80
70
60
50
40
30
20
10
0
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+
NZB;NZW Females
NZB;NZW Males

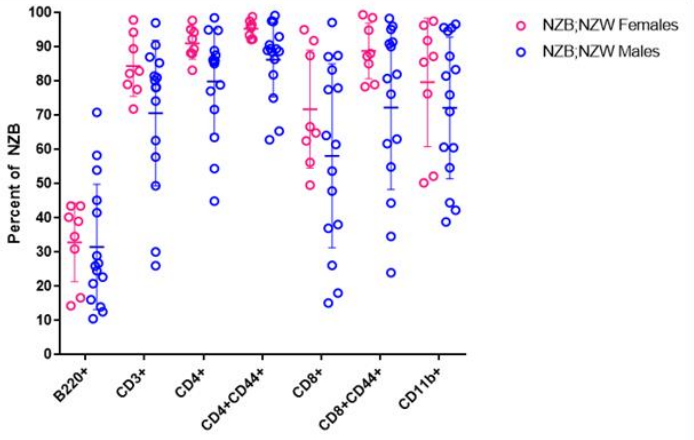
K


Spleen
Percent of Splenocytes
100
90
80
70
60
50
40
30
20
10
0
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+
N
11 NZW
9 NZB
13 NZB/NZW F1
40 NZB;NZW Non-Hematopoietic
24 NZB;NZW Chimera (NZB)
NZB;NZW Chimera (NZW)

L


Percentage of Lymphocytes from Parent Strain
100
80
60
40
20
0
****
****
B220+
CD3+
NZW;B6 (NZW)
NZB;B6 (NZB)
NZB;NZW Female (NZB)
NZB;NZW Female (NZW)
NZB;NZW Male (NZB)
NZB;NZW Male (NZW)

M

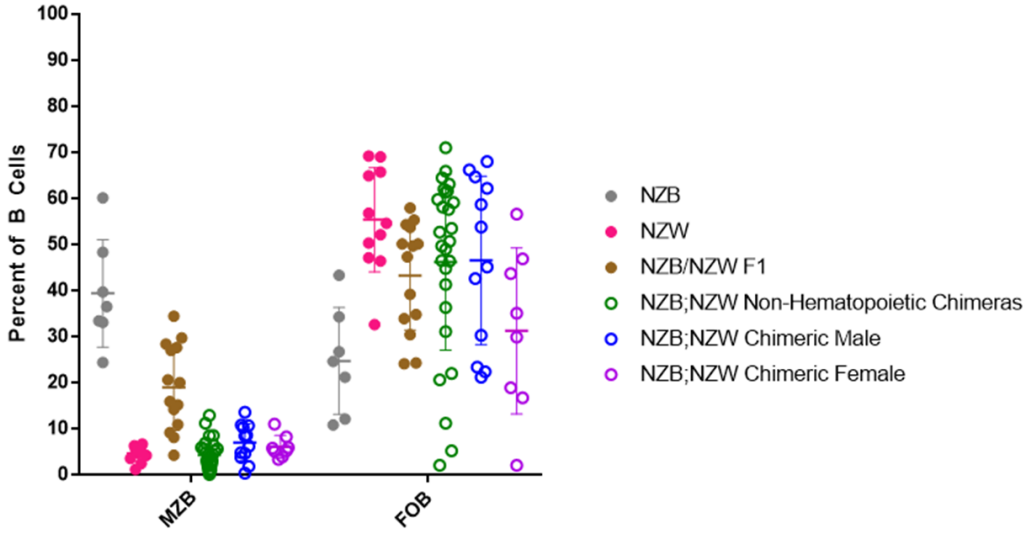

Percent of NZB
100
90
80
70
60
50
40
30
20
10
0
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+
NZB;NZW Females
NZB;NZW Males

Marginal Zone B (MZB) Follicular B (FoB), and T Follicular Helper (T_{FH}) Cells

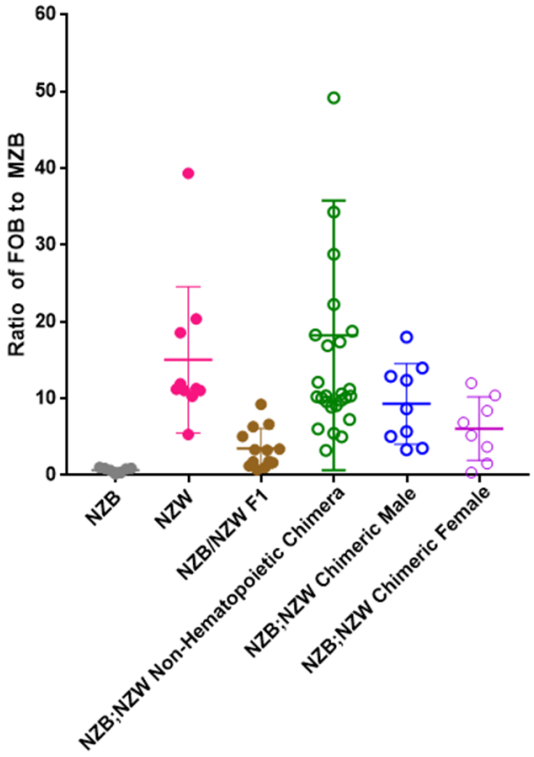
Within the spleen special populations of cells including MZB, FoB, and TFH are important for mounting immune responses. In comparison to other mouse strains, NZB mice show a high proportion at MZB cells, and thus a reduced proportion of FoB cells (Figure 5A, B). This finding has previously been reported in the literature as well [9]. As a result, the ratio of FoB to MZB cells is on average 0.67, whereas for NZW and B6 it is 15.03 and 21.37, respectively. NZB/NZW F1, NZB;NZW chimeric females, and NZB;NZW chimeric males all have more MZB than NZW, leading to a decrease it the FoB to MZB ratio, with averages of 3.50, 6.06, and 9.28 respectively. Finally, the NZB;NZW non-hematopoietic chimeras have a frequency of MZB similar to NZW, with a FoB to MZB ratio of 18.2. This is expected as the immune cells in the NZB;NZW non-hematopoietic chimeras are all derived from the NZW strain. As NZB wildtype mice have been shown to have a larger population of MZB cells, in the NZB;NZW chimeric mice the majority (~80%) of the MZB cells are of NZB origin, whereas the majority of the FoB cells (~60%) are from NZW. Of the B cells studied in the NZB;NZW chimeras thus far, this is the only population of B cells that mainly comes from NZB. Whether this expansion of NZB MZB cells is important for SLE pathogenesis is unknown. While alone it does not promote SLE in the wildtype NZB mice, it is possible it may play a role in the chimeric or hybrid environment with NZW. The TFH cells in the NZB;NZW hematopoietic chimeras are also found at higher frequencies than in NZW, NZB, or NZB;NZW non-hematopoietic chimeras, and they mostly come from NZB origin (~90%) (Figure 5C, D).

Figure 5. NZB;NZW Chimeras Have Disproportionate T and B Cell Contribution in the Spleen. (A) Percent of MZB and FoB of B cells. (B) Ratio of the MZB to FoB in the spleens. (C) Individual values of the percent of each cell population that comes from NZB origin. (D) Percent of TFH of T cells. *** = $p \leq 0.001$, N.S. = Not Significant $p > 0.05$ using an unpaired T test.

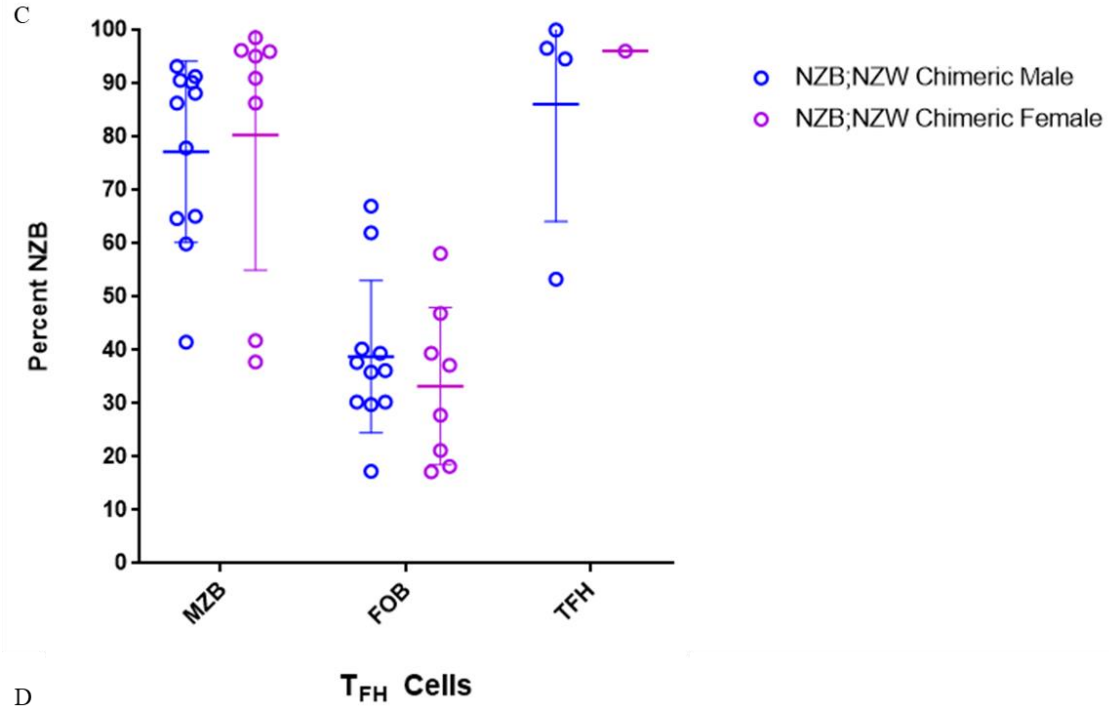
A


Percent of B Cells
100
90
80
70
60
50
40
30
20
10
0
MZB
FOB
NZB
NZW
NZB/NZW F1
NZB;NZW Non-Hematopoietic Chimeras
NZB;NZW Chimeric Male
NZB;NZW Chimeric Female

B

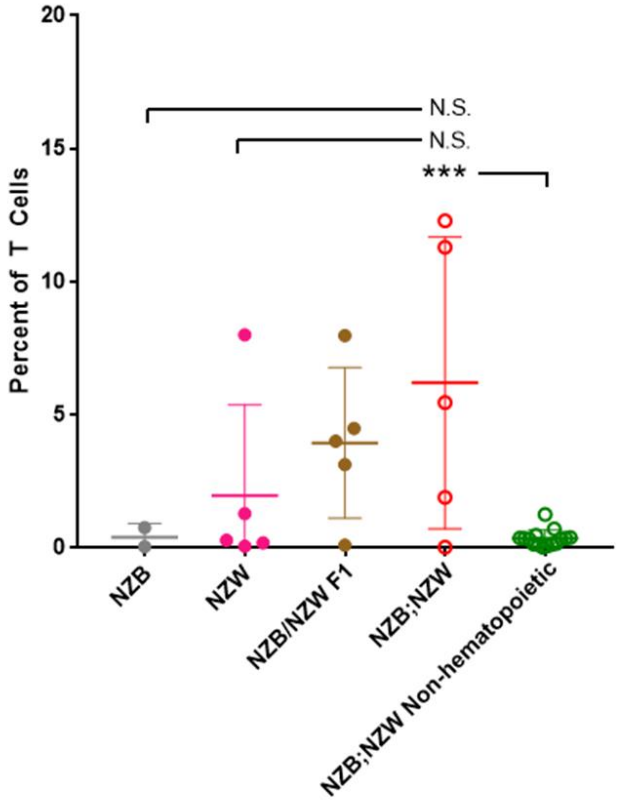

Ratio of FOB to MZB
60
50
40
30
20
10
0
NZB
NZW
NZB/NZW F1
NZB;NZW Non-Hematopoietic Chimera
NZB;NZW Chimeric Male
NZB;NZW Chimeric Female

C


Percent NZB
100
90
80
70
60
50
40
30
20
10
0
MZB
FOB
TFH
NZB;NZW Chimeric Male
NZB;NZW Chimeric Female

T_{FH} Cells

D


Percent of T Cells
20
15
10
5
0
N.S.
N.S.
***
NZB
NZW
NZB/NZW F1
NZB;NZW
NZB;NZW Non-hematopoietic

Peritoneal Fluid (PF) B Cell Subsets

In the PF there are B cell subsets including B-1a, B-1b, and B-2 cells that have important roles in immunity. B-1a cells have been identified as a potential reservoir of low-affinity self-reactive cells that could be the precursors of the autoreactive B cells in SLE. There was no difference in the overall frequencies of the B cells compared to wildtype of NZB;NZW non-hematopoietic mice (Figure 6A). Furthermore, just as is seen in the periphery, the majority of B cells are from the NZW origin (Figure 6B).

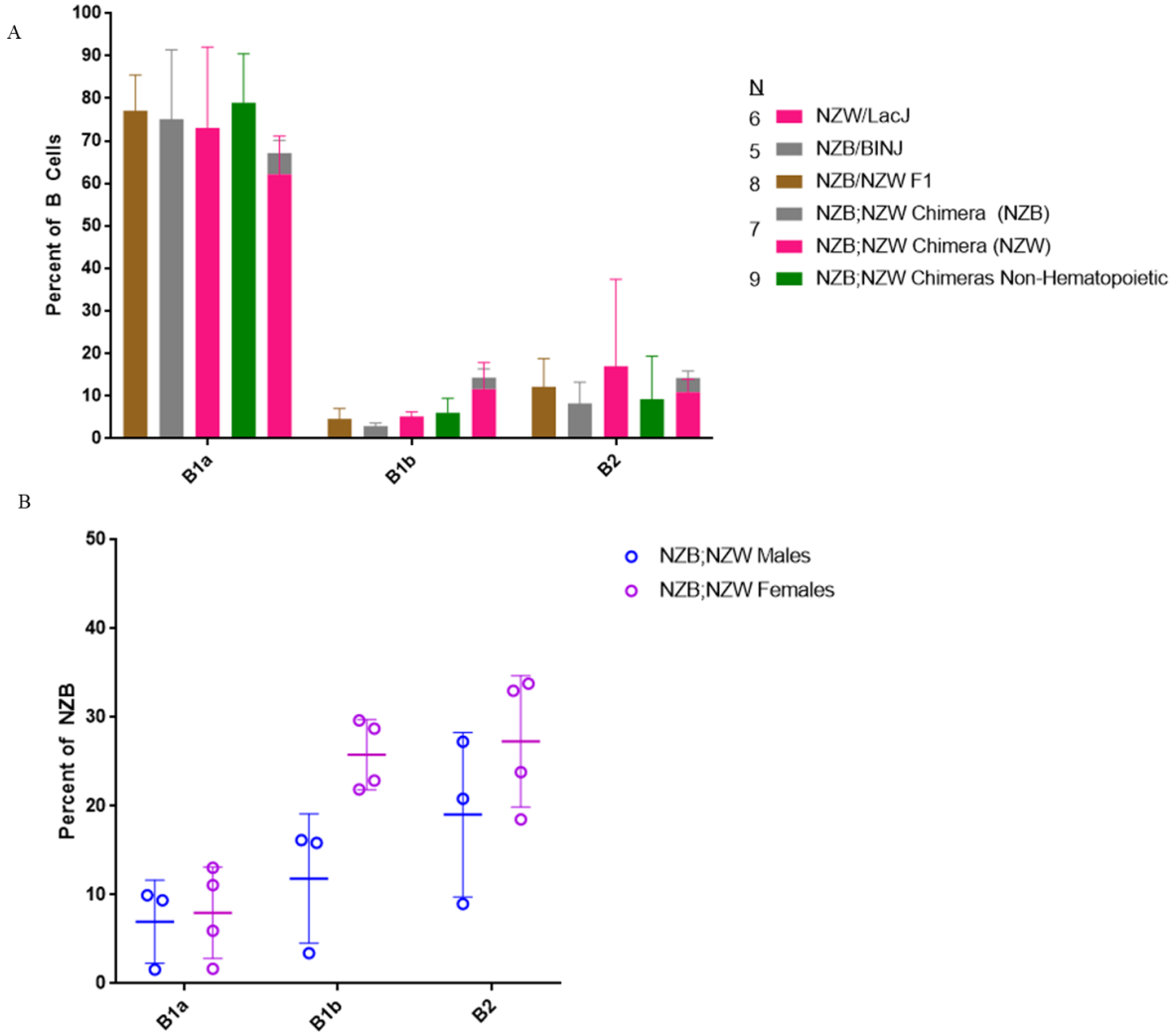


Figure 6. B Cell Subsets in the Peritoneal Fluid of NZB;NZW Chimeras

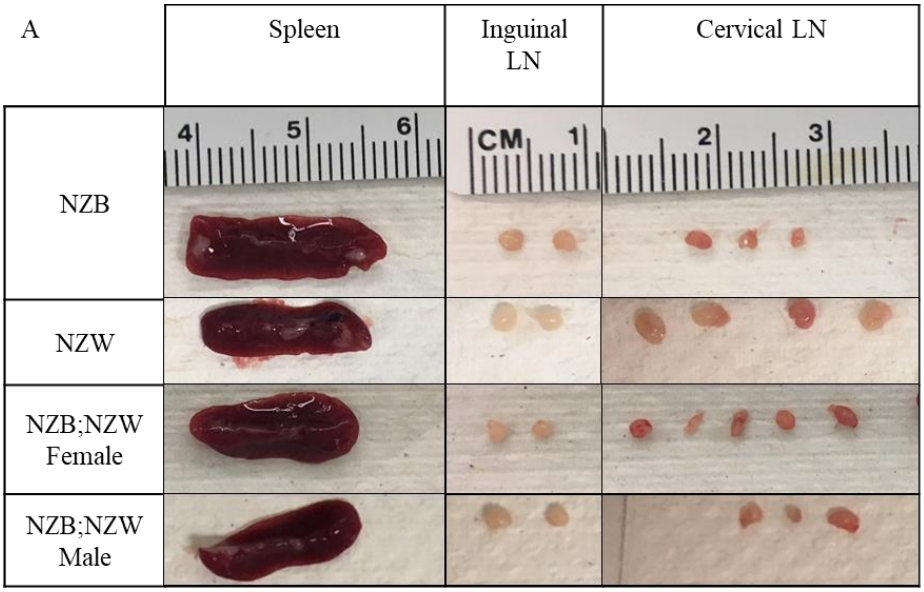
(A) Averaged percent of cell populations in total leukocytes for wildtype mice and NZB;NZW Chimeras at 8-9 months of age. Stacked bars represent the combined total of NZB and NZW cells within the chimeras. (B) Individual values of the percent of each cell population that comes from NZB origin.

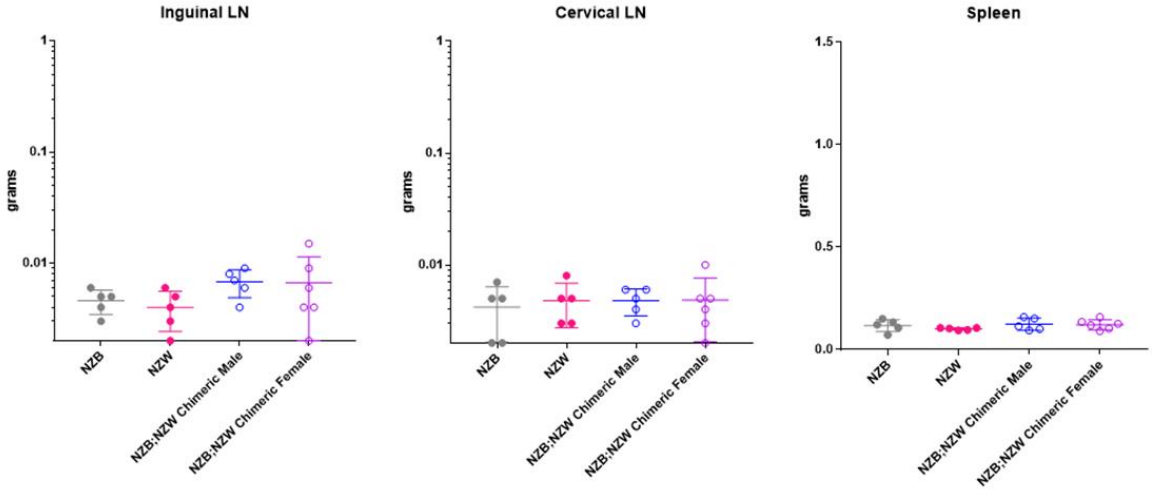
The Autoimmune Phenotype of Young NZB;NZW Chimeras

In order to identify early cell processes involved in disease, NZB;NZW chimera cell populations were identified at 2 months of age, prior to most disease features. At this age, these mice did not develop lymphadenopathy or splenomegaly (Figure 7A). Similarly to the mice at 8 month of age, there was no difference between the overall percentages of major cell populations between the NZB;NZW chimeras and controls (Figure 7B, E, H, K), and the majority of the B cells were of NZW origin, while the majority of T cells were from the NZB origin (Figure 7D, G, J, M). However, unlike the 8 month old mice, the unequal distribution of NZB to the different cell types was equally apparent in both the NZB;NZW chimeric males and females.

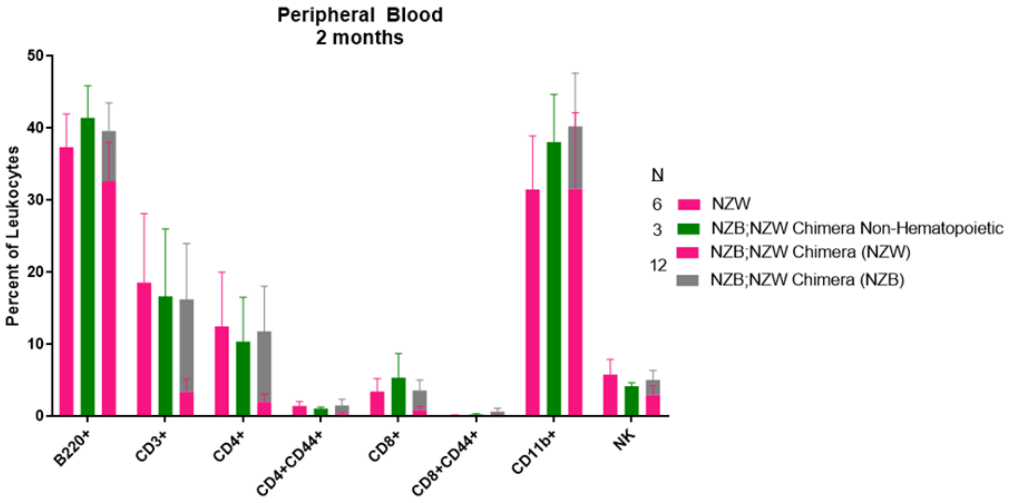
Figure 7. Young NZB;NZW Chimeras Have Disproportionate T and B Cell Contribution to Major Immune Cell Subsets.

(A) Spleens, inguinal LN, and cervical LN were collected from 2 month old mice and weighed (g). (B, E, H, K) Averaged percent of cell populations in total leukocytes for wildtype mice and NZB;NZW chimeras at 2 months of age. Stacked bars represent the combined total of NZB and NZW cells within the chimeras. (C, F, I, L) Individual value of the percent of T or B cells in the cells of the parental strain. (D, G, J, M) Percent of each cell population that comes from NZB origin. *** = $p \leq 0.001$, **** = $p \leq 0.0001$ using multiple T tests.

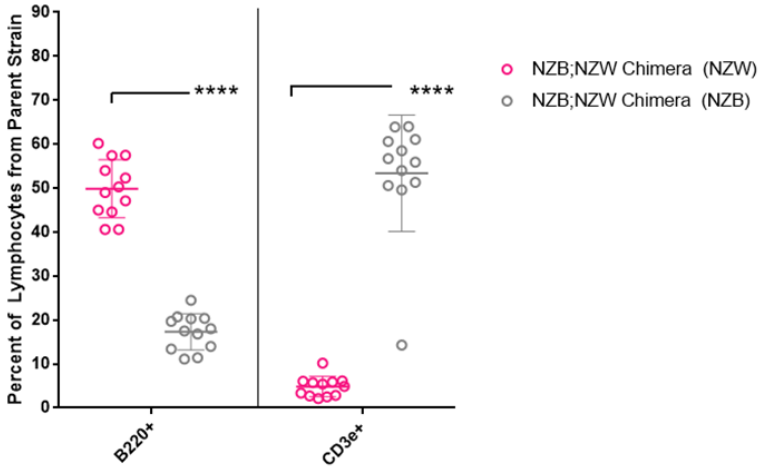

A
Spleen
Inguinal LN
Cervical LN
NZB
NZW
NZB;NZW Female
NZB;NZW Male


Inguinal LN
Cervical LN
Spleen
grams
NZB
NZW
NZB;NZW Chimeric Male
NZB;NZW Chimeric Female

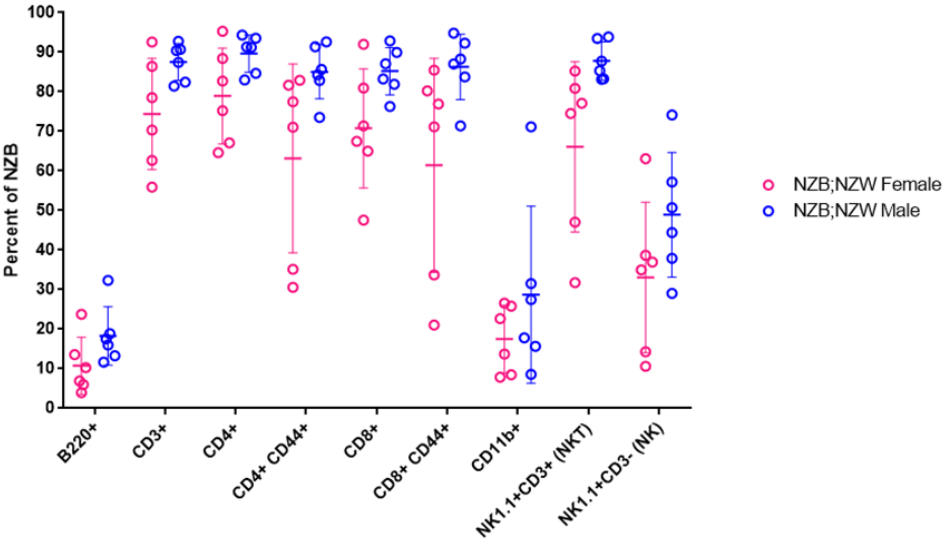
B


Peripheral Blood
2 months
Percent of Leukocytes
50
40
30
20
10
0
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+
NK
N
6
3
12
NZW
NZB;NZW Chimera Non-Hematopoietic
NZB;NZW Chimera (NZW)
NZB;NZW Chimera (NZB)

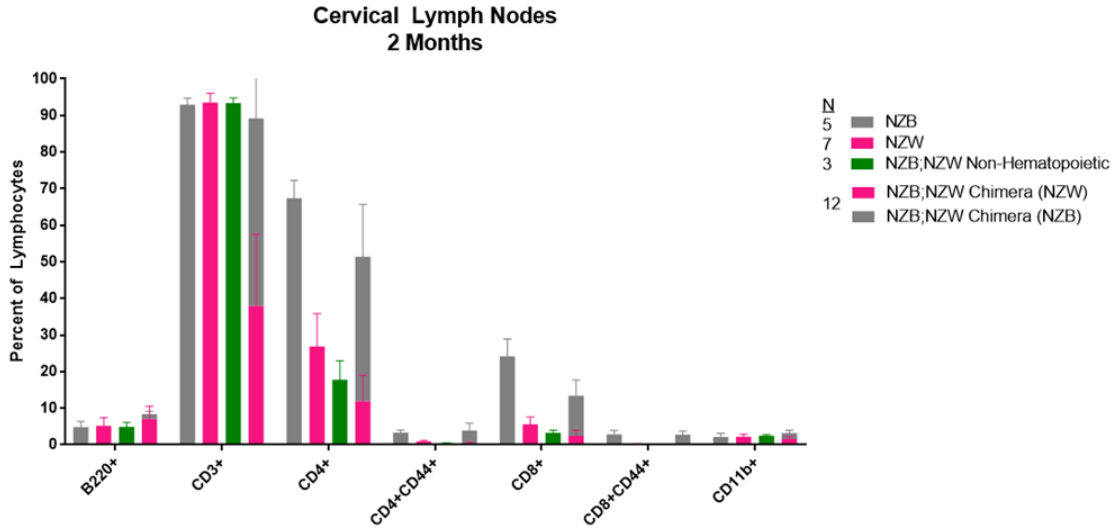
C


Percent of Lymphocytes from Parent Strain
90
80
70
60
50
40
30
20
10
0
****
****
B220+
CD3e+
NZB;NZW Chimera (NZW)
NZB;NZW Chimera (NZB)

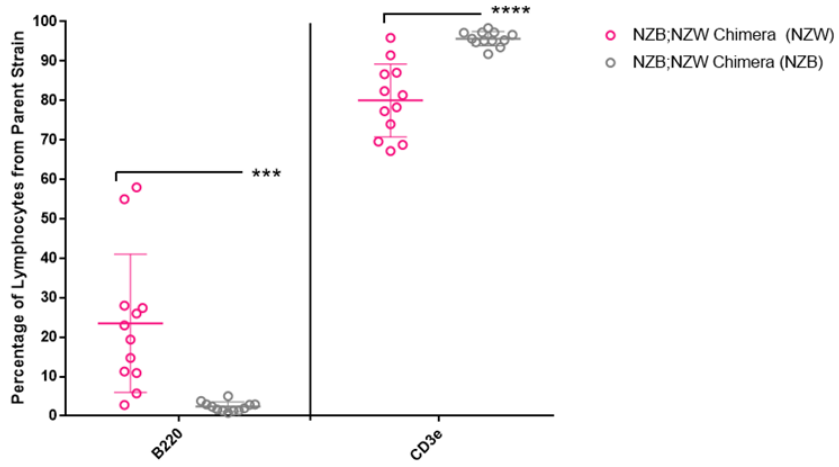
D


Percent of NZB
100
90
80
70
60
50
40
30
20
10
0
B220+
CD3+
CD4+
CD4+ CD44+
CD8+
CD8+ CD44+
CD11b+
NK1.1+CD3+ (NKT)
NK1.1+CD3- (NK)
NZB;NZW Female
NZB;NZW Male

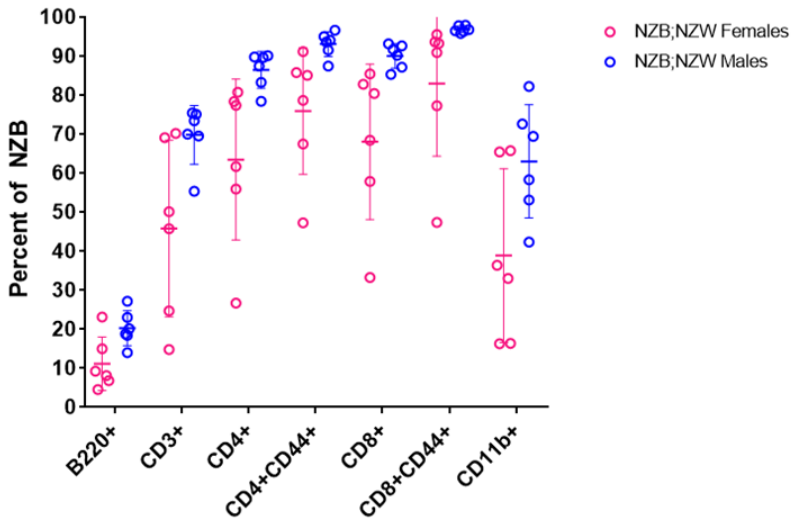
E


Cervical Lymph Nodes
2 Months
Percent of Lymphocytes
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+
N
5 NZB
7 NZW
3 NZB;NZW Non-Hematopoietic
12 NZB;NZW Chimera (NZW)
NZB;NZW Chimera (NZB)

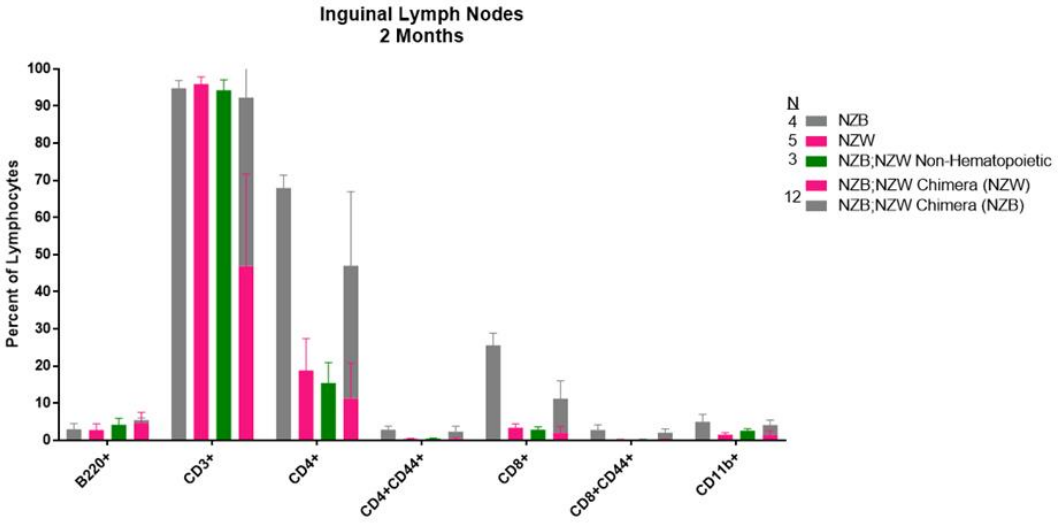
F


Percentage of Lymphocytes from Parent Strain
***
****
B220
CD3e
NZB;NZW Chimera (NZW)
NZB;NZW Chimera (NZB)

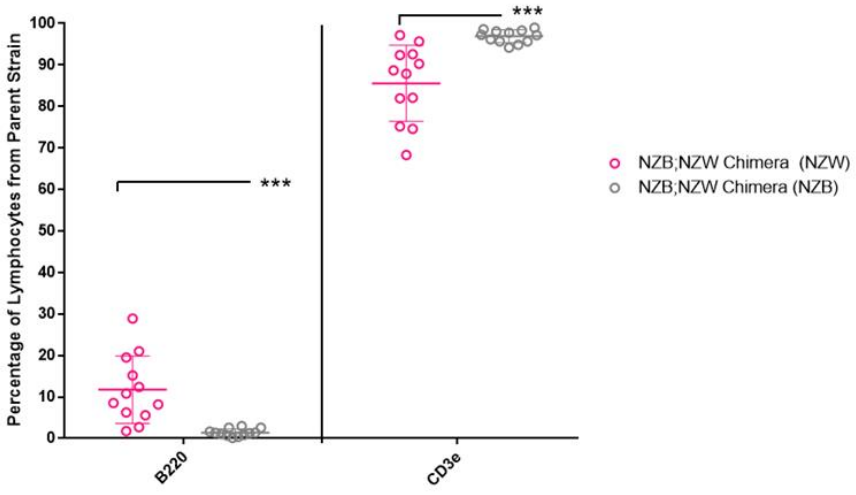
G


Percent of NZB
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+
NZB;NZW Females
NZB;NZW Males

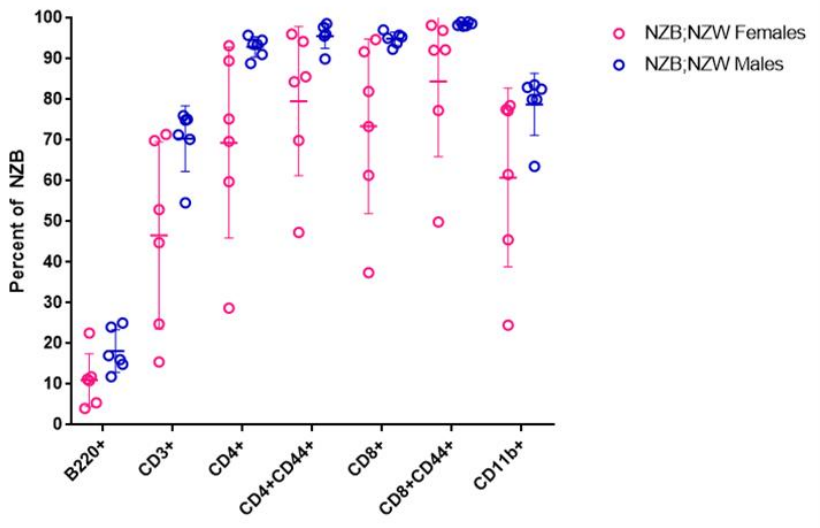
H


Inguinal Lymph Nodes
2 Months
Percent of Lymphocytes
N
4 NZB
5 NZW
3 NZB;NZW Non-Hematopoietic
12 NZB;NZW Chimera (NZW)
NZB;NZW Chimera (NZB)
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+

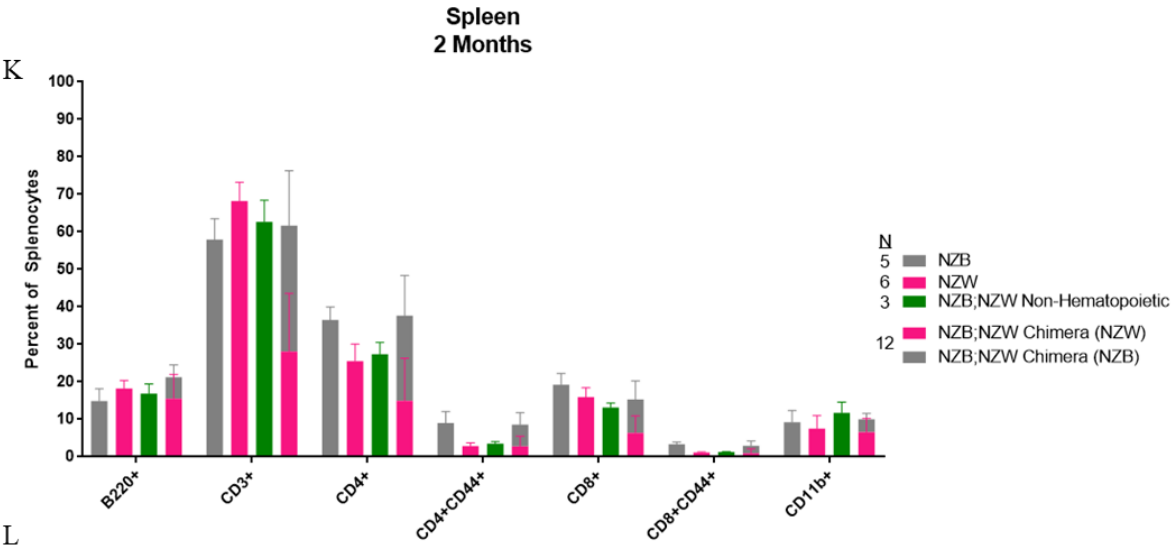
I


Percentage of Lymphocytes from Parent Strain
***
***
NZB;NZW Chimera (NZW)
NZB;NZW Chimera (NZB)
B220
CD3e

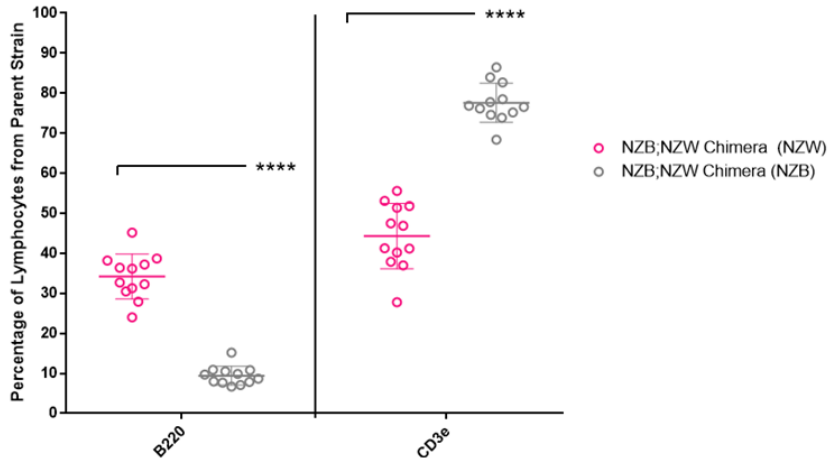
J


Percent of NZB
NZB;NZW Females
NZB;NZW Males
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+

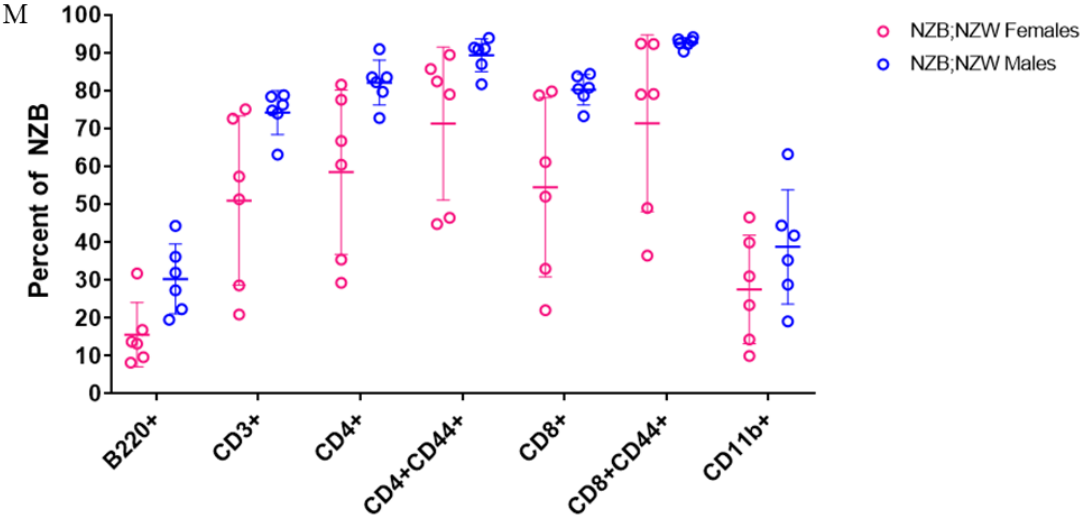
K


Spleen
2 Months
Percent of Splenocytes
100
90
80
70
60
50
40
30
20
10
0
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+
N
5 NZB
6 NZW
3 NZB;NZW Non-Hematopoietic
12 NZB;NZW Chimera (NZW)
NZB;NZW Chimera (NZB)

L


Percentage of Lymphocytes from Parent Strain
100
90
80
70
60
50
40
30
20
10
0
****
****
B220
CD3e
NZB;NZW Chimera (NZW)
NZB;NZW Chimera (NZB)

M


Percent of NZB
100
90
80
70
60
50
40
30
20
10
0
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+
NZB;NZW Females
NZB;NZW Males

Additional T Cell Subsets

In addition to the CD4+ and CD8+ T cell subsets analyzed in the PB, spleen, and LN, there are several other T cell subsets that play a role in autoimmunity, including TH17, TH1, TH2, TFH, and Treg. The frequency of each of these cell populations were tested in the 2 month old chimeras. The percentage of TFH in the spleen was significantly greater in NZB;NZW chimeras than either wildtype parental strain, and this increase was not apparent in non-hematopoietic NZB;NZW chimeras (Figure 8A). Furthermore, most of these cells were of NZB origin (Figure 8B). The frequency of other subsets, such as TH17, TH1, TH2, and Tregs in the spleen and LN were similar to that seen in the parental strains (Figure 8A), and these mostly were derived from NZB (Figure 8B).

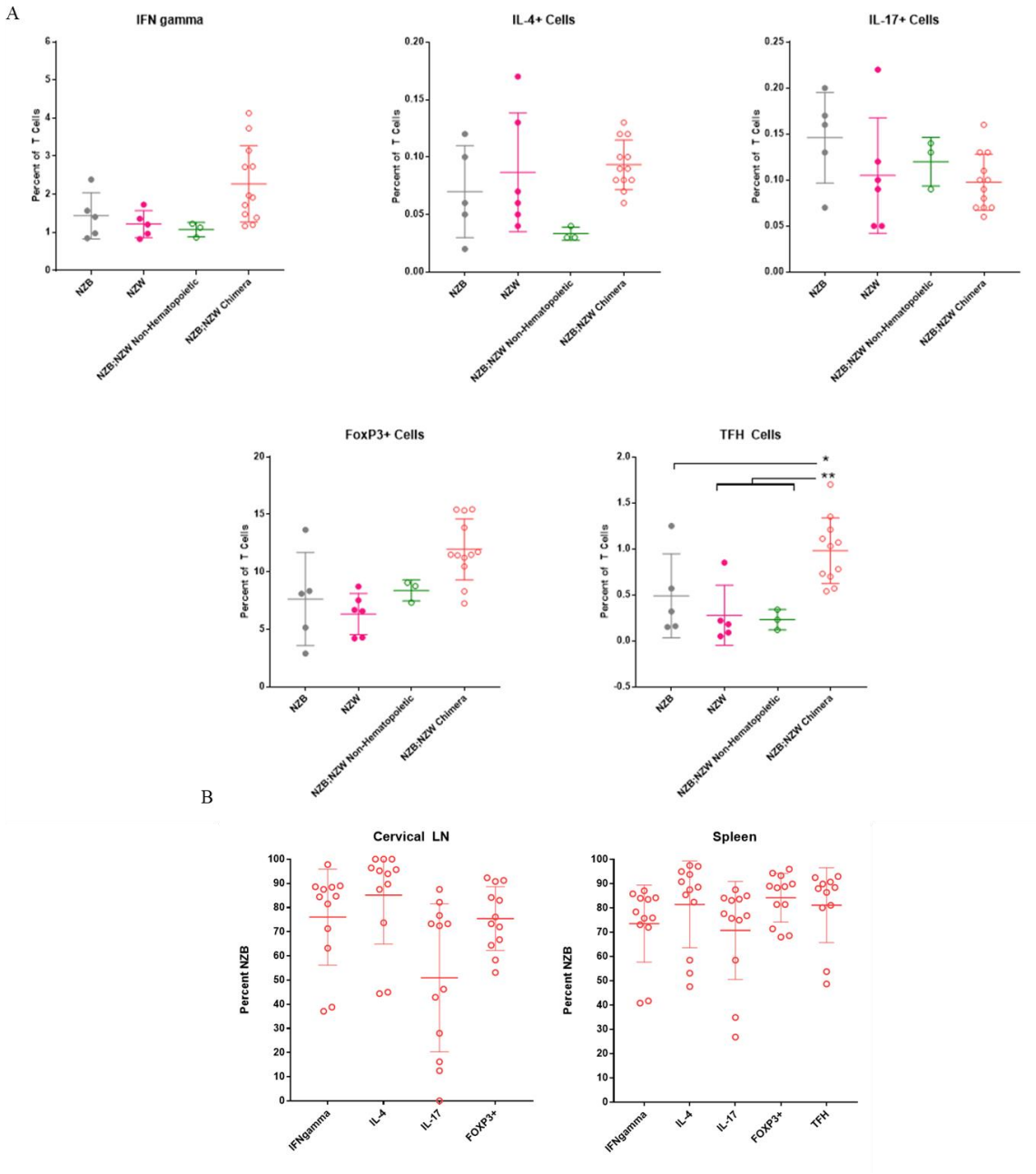


Figure 8. TFH, TH17, TH1, TH2, and Treg Populations in Young NZB;NZW Chimeras

(A) Individual percent of T cell subsets in T cells in wildtype mice and NZB;NZW Chimeras at 2 months of age (B) Individual values of the percent of each cell population that come from NZB origin in NZB;NZW chimeras. * = $p \leq 0.05$, ** = $p \leq 0.01$ using an unpaired T test.

The Autoimmune Phenotype of NZB$^{Cd3e-/-}$;NZW Chimeras

To identify what cell may be involved in the disease process, every cell type analyzed was correlated with different parameters of disease, including class score, organ weights, and autoantibody production. Consistently it was observed that these parameters of disease positively correlated with NZB T cells, especially CD4+ cells and CD4+ CD44+ cells (Figure 9A, B, C, D). Because NZB T cells were strongly and consistently correlated with anti-DNA antibody titer, we created a *Cd3e* knockout in NZB ESC prior to injecting these cells into NZW blastocysts. The resulting mice were not lymphopenic and most hematopoietic cell populations were derived from both strains. However, all the T cells were of NZW origin (Figure 10A, B). The NZB$^{Cd3e-/-}$; NZW chimeras were then characterized similarly to the NZB;NZW chimeras to determine if there were any differences in the SLE phenotype.

Autoantibody Production

Unlike the NZB;NZW chimeras, only 14% of the mice developed IgG anti-dsDNA antibodies (Figure 10C) or other autoantibody specificities (Figure 10D, Appendix B). Of the NZB$^{Cd3e-/-}$; NZW chimeras that developed autoimmunity, 75% (3/4) were female, compared to 44% (11/25) of nonautoimmune mice. However, this occurred 2-3 months later than in the wildtype NZB;NZW chimeras.

Lymphoid Organ Pathology

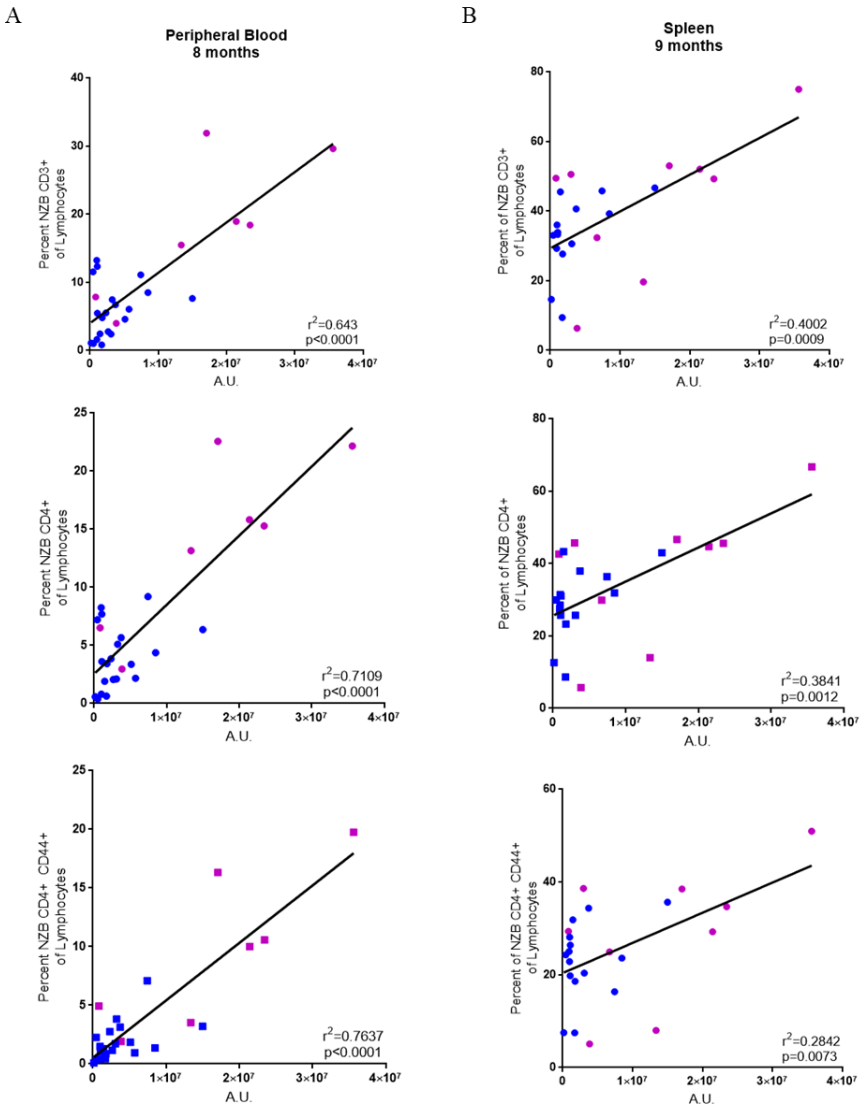
Moreover, none of the NZB$^{Cd3e-/-}$; NZW chimeras had lymphadenopathy or splenomegaly, and all organ weights match those of the parental controls (Figure 10E). H&E sections of cervical lymph nodes from NZB$^{Cd3e-/-}$; NZW chimeras show scant paracortex, rare follicle formation, and relatively normal medullary cords compared to controls (Figure 10F).

Kidney Pathology

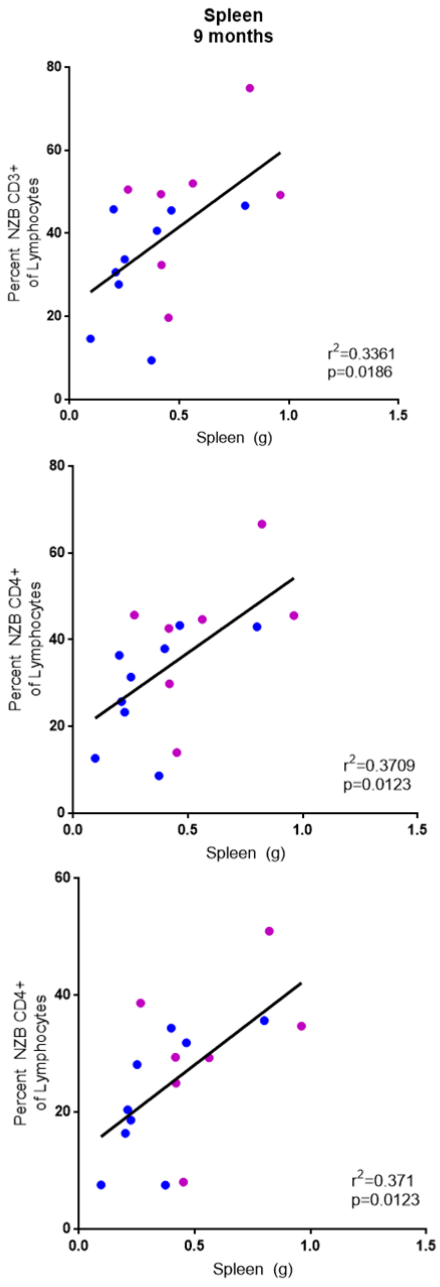
Furthermore, the NZB$^{Cd3e-/-}$; NZW chimeras do not develop glomerulonephritis (Figure 10G). Of the mice that did not produce autoantibodies, the NZB$^{Cd3e-/-}$; NZW chimeras had significantly reduced proteinuria compared to NZB;NZW chimeras (Figure 10I). NZB$^{Cd3e-/-}$; NZW chimeras had an average of 1.4 mg protein/mg creatinine and 0.6mg/mg at 4 and 6 months, respectively. While NZB;NZW chimeras had an average of 7.4 mg/mg and 3.2mg/mg at 4 and 6 months, respectively. Furthermore NZB$^{Cd3e-/-}$; NZW chimeras had reduced activity/chronicity of glomerulonephritis, with an average activity/chronicity score of 1.6, compared to the average activity/chronicity score of 6.2 for NZB;NZW female chimeras. (Figure 10H) (Table 6).

Figure 9. Correlation of NZB CD3e+ Subsets with Disease.

Anti-dsDNA autoantibody production (A.U.) was correlated with the percentage of different NZB T cell subsets in the PB (A) and spleen (B). The weight of the spleen (g) was correlated with the percentage of different NZB T cell subsets in the spleen (C), and the weight of the cervical LN (g) was correlated with the percentage of different NZB T cell subsets in the cervical LN (D). Females are indicated in purple and males are indicated in blue. Line is the line of best fit and r^2 value is shown within each graph.



C


Spleen
9 months
Percent NZB CD3+ of Lymphocytes
r²=0.3361
p=0.0186
Spleen (g)
Percent NZB CD4+ of Lymphocytes
r²=0.3709
p=0.0123
Spleen (g)
Percent NZB CD4+ of Lymphocytes
r²=0.371
p=0.0123
Spleen (g)

D

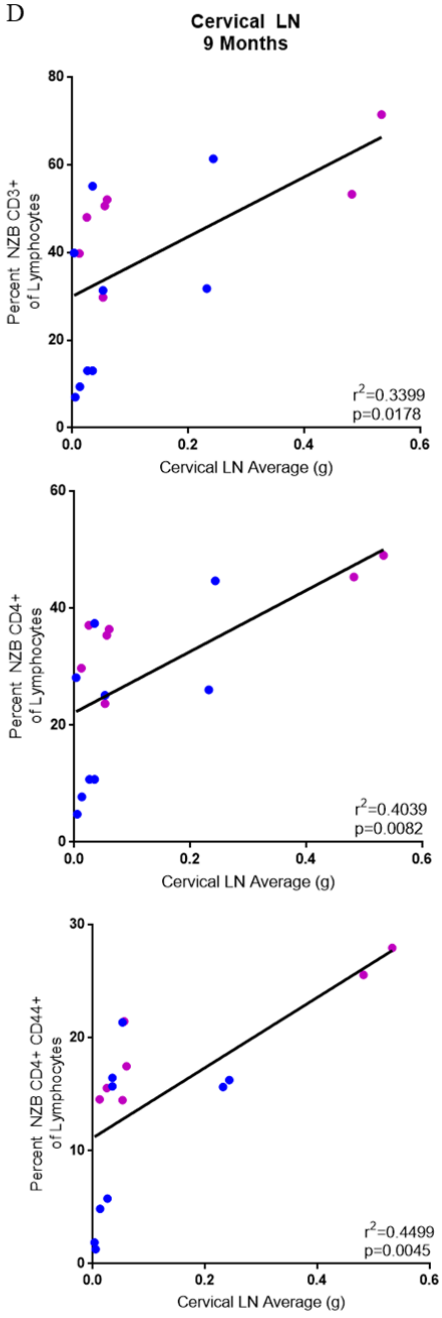
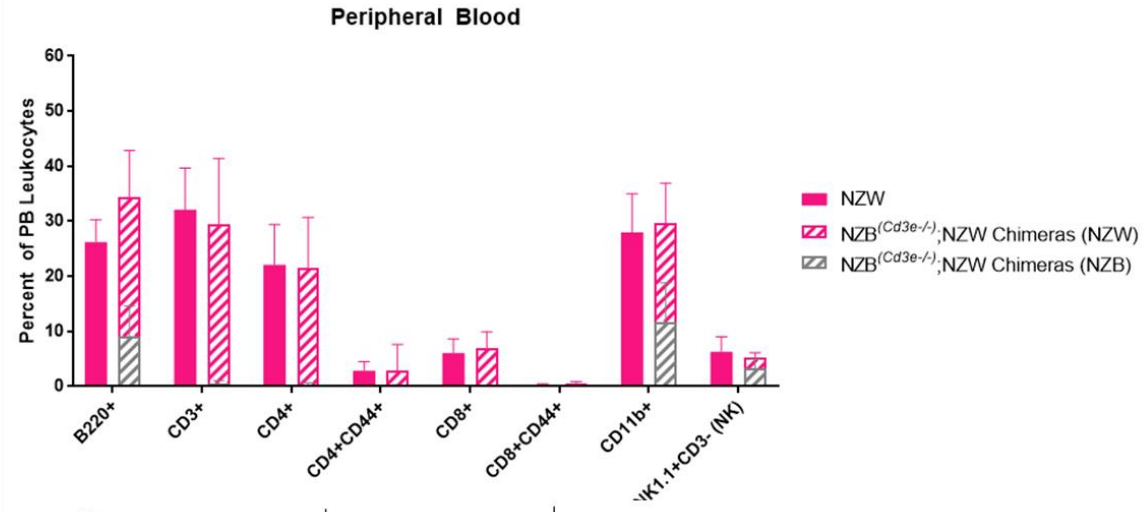

Cervical LN
9 Months
Percent NZB CD3+ of Lymphocytes
r²=0.3399
p=0.0178
Cervical LN Average (g)
Percent NZB CD4+ of Lymphocytes
r²=0.4039
p=0.0082
Cervical LN Average (g)
Percent NZB CD4+ CD44+ of Lymphocytes
r²=0.4499
p=0.0045
Cervical LN Average (g)

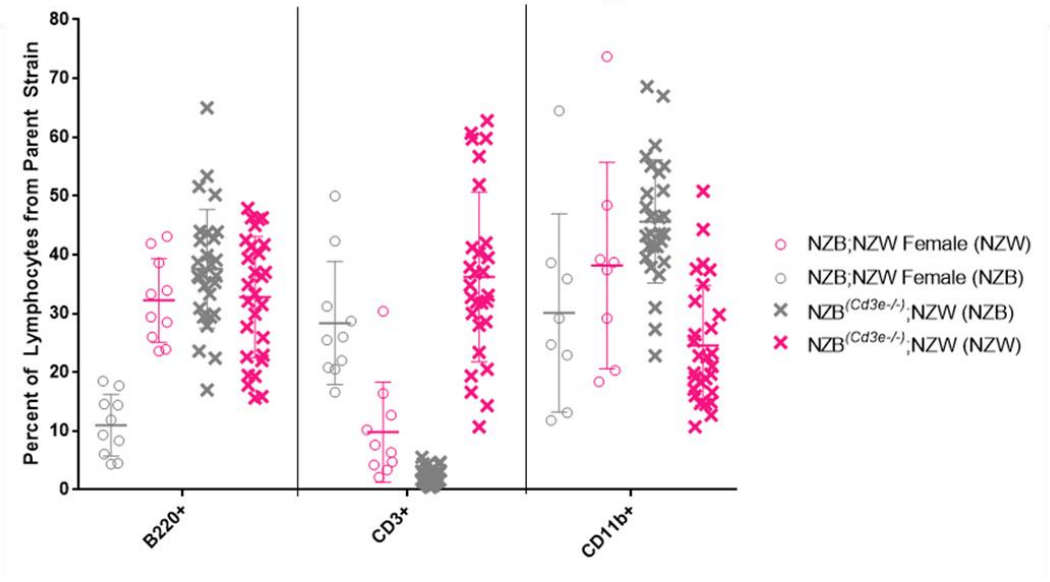
Figure 10. NZB T Cells Are Necessary for Strong Autoimmunity.

(A) Averaged percent of cell populations in total leukocytes for at 3 months of age. Stacked bars represent the combined total of NZB and NZW cells within the chimeras. (B) Individual value of the percent of T, B, or CD11b+ cells in the cells of the parental strain. (C) Individual values for the activity level of IgG anti-dsDNA antibodies in different mouse strains over time. (D) Serum from mice were collected and analyzed on a peptide microarray for IgM and IgG isotypes. For the heatmap, values greater than the median are red, values at the median are black, and values below the median are green. (E) Spleens, inguinal LN, and cervical LN were collected from 7 month old $NZB^{CD3e-/-}$;NZW mice and weighed (g). Representative images of organs are shown below. (F) H&E stained cervical LN from 7 month old $NZB^{CD3e-/-}$;NZW mice. (G) Representative image of various stains from the kidneys collected from the mice. (H) Activity and chronicity index of each mouse. (I) Urine was collected monthly and tested for total protein and creatinine using Vitros. NZB/NZW F1, NZB/BINJ, and NZW/LacJ mice are $\geq$9 months of age. * = $p \leq 0.05$,** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$ using the Mann-Whitney test.

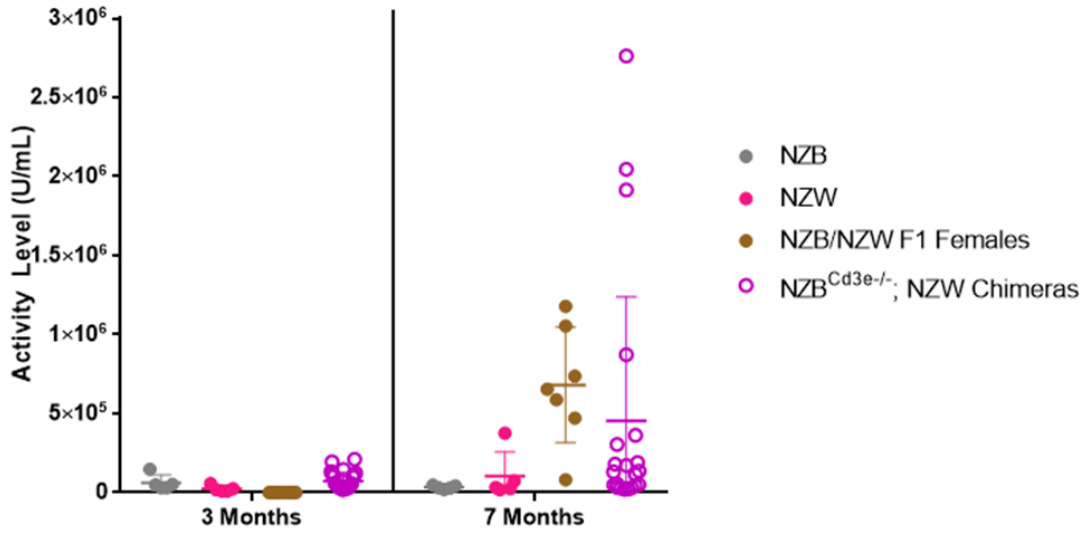
A


Peripheral Blood
Percent of PB Leukocytes
60
50
40
30
20
10
0
B220+
CD3+
CD4+
CD4+CD44+
CD8+
CD8+CD44+
CD11b+
NK1.1+CD3- (NK)
NZW
NZB(Cd3e-/-);NZW Chimeras (NZW)
NZB(Cd3e-/-);NZW Chimeras (NZB)

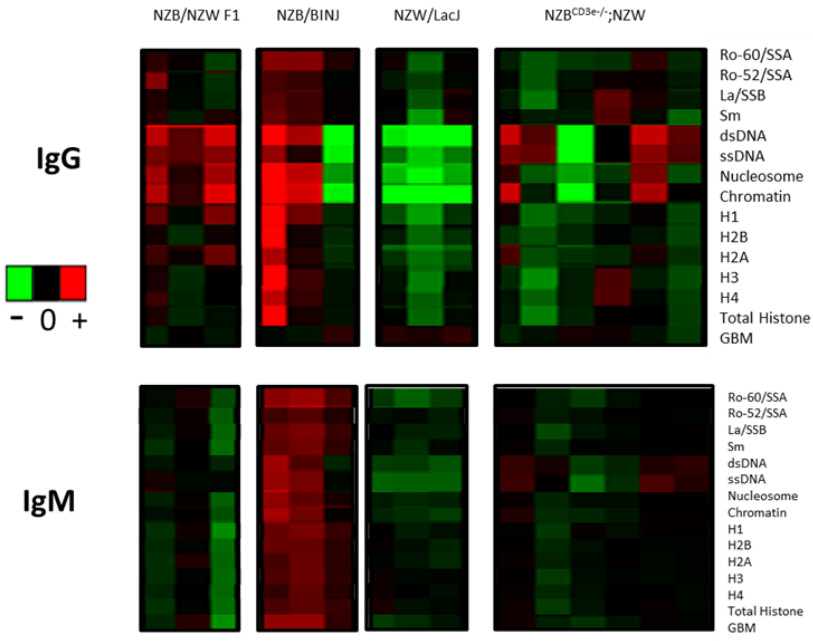
B


Percent of Lymphocytes from Parent Strain
80
70
60
50
40
30
20
10
0
B220+
CD3+
CD11b+
NZB;NZW Female (NZW)
NZB;NZW Female (NZB)
NZB(Cd3e-/-);NZW (NZB)
NZB(Cd3e-/-);NZW (NZW)

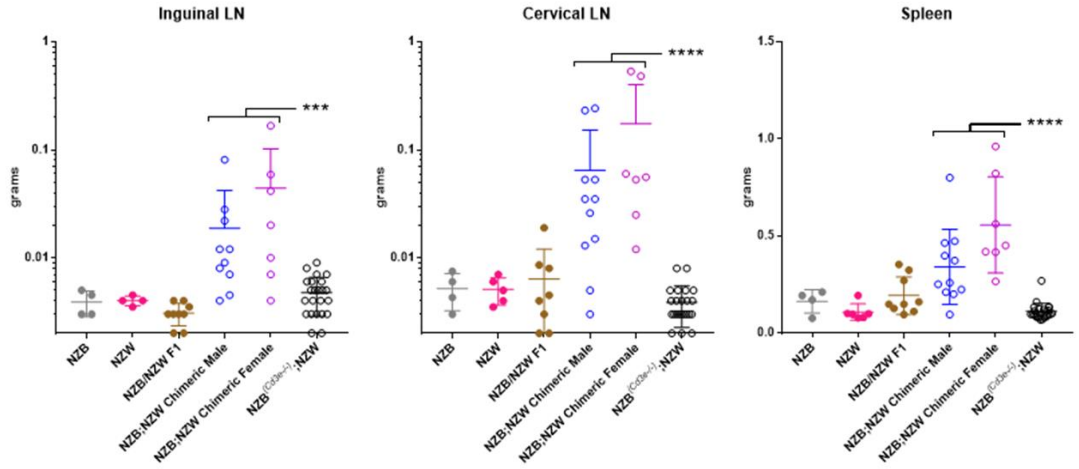
C

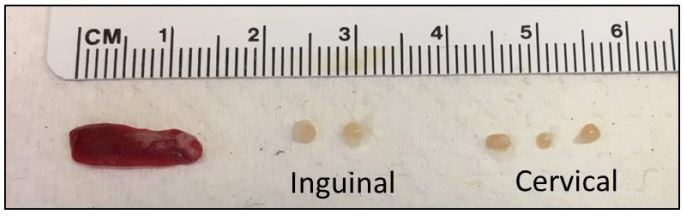

3×10⁶
2.5×10⁶
2×10⁶
1.5×10⁶
1×10⁶
5×10⁵
0
Activity Level (U/mL)
3 Months
7 Months
NZB
NZW
NZB/NZW F1 Females
NZB Cd3e-/-; NZW Chimeras

D

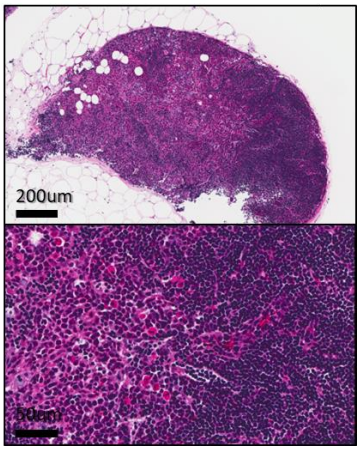

NZB/NZW F1
NZB/BINJ
NZW/LacJ
NZB CD3e-/-;NZW
IgG
- 0 +
Ro-60/SSA
Ro-52/SSA
La/SSB
Sm
dsDNA
ssDNA
Nucleosome
Chromatin
H1
H2B
H2A
H3
H4
Total Histone
GBM
IgM
Ro-60/SSA
Ro-52/SSA
La/SSB
Sm
dsDNA
ssDNA
Nucleosome
Chromatin
H1
H2B
H2A
H3
H4
Total Histone
GBM

E

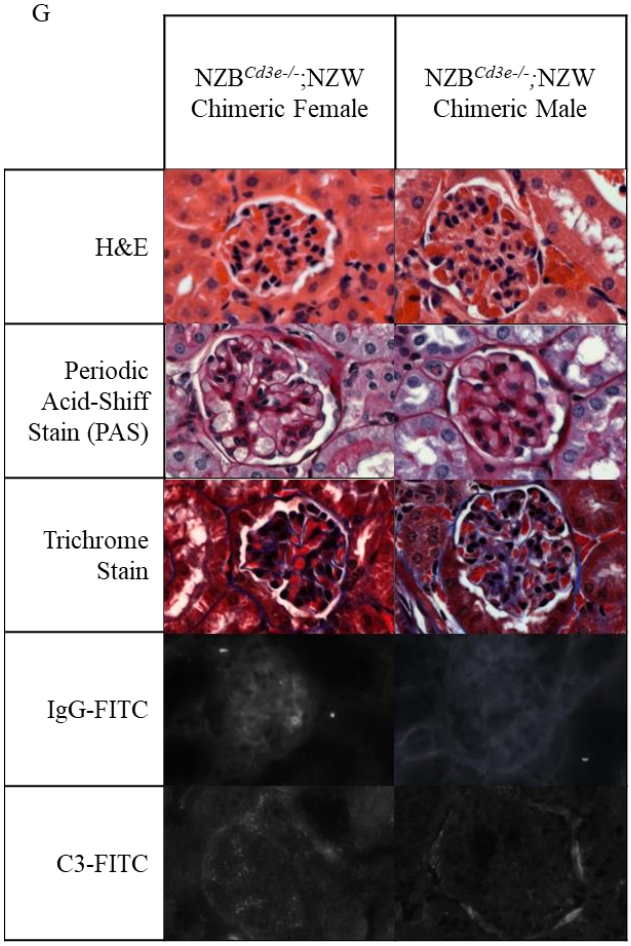

Inguinal LN
Cervical LN
Spleen
grams
***
****
****
NZB
NZW
NZB/NZW F1
NZB:NZW Chimeric Male
NZB:NZW Chimeric Female
NZB (Cd3e-/-);NZW


CM
1
2
3
4
5
6
Inguinal
Cervical

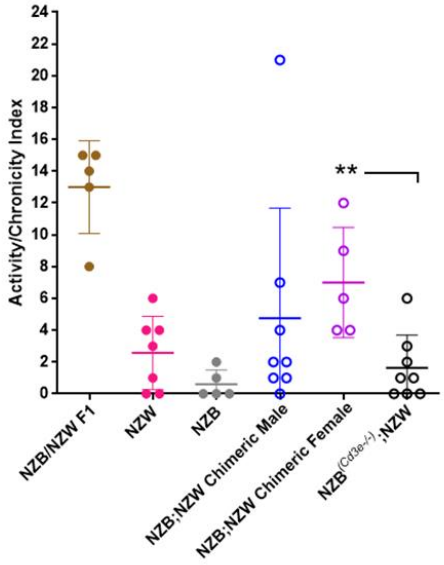
F


200um
50um

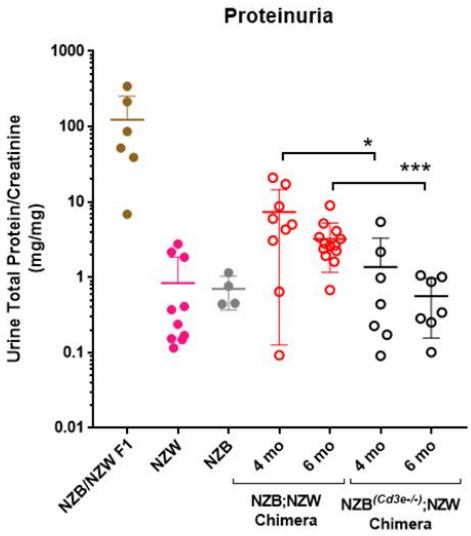
G


NZB$^{Cd3e-/-}$;NZW Chimeric Female
NZB$^{Cd3e-/-}$;NZW Chimeric Male
H&E
Periodic Acid-Shiff Stain (PAS)
Trichrome Stain
IgG-FITC
C3-FITC

H


Activity/Chronicity Index
**
NZB/NZW F1
NZW
NZB
NZB;NZW Chimeric Male
NZB;NZW Chimeric Female
NZB$^{(Cd3e-/-)}$;NZW

I


Proteinuria
Urine Total Protein/Creatinine (mg/mg)
*
***
NZB/NZW F1
NZW
NZB
4 mo
6 mo
4 mo
6 mo
NZB;NZW Chimera
NZB$^{(Cd3e-/-)}$;NZW Chimera

CHAPTER SIX
Results

ABERRANT TOLERANCE IN NZB;NZW CHIMERAS

Overview

In humans and many mouse models of SLE, it is unknown why individuals break tolerance and develop autoimmunity. Understanding why this occurs provides insight into potential therapeutic targets or ways to prevent the onset. While it is apparent that the NZB;NZW chimeras develop autoimmunity, further research was completed to determine how this autoreactivity occurs.

Loss of Self-Tolerance

To determine which strain was able to produce these autoantibodies, B cell autoreactivity to dsDNA was tested using ELIspot (Figure 11A). The C57BL/6J showed significantly fewer IgG anti-dsDNA B cells than NZB/NZW F1 hybrid mice or NZB;NZW chimeras. Furthermore, both the NZB and NZW B cells from the NZB;NZW chimeras produced IgG anti-dsDNA and were found at frequencies similar to those of the NZB/NZW F1 hybrid mice. This indicates that B cells of both parental strains can lose tolerance to self in the chimeric environment.

Since NZB and NZW develop together from early embryonic stages, tolerance between the two MHC-disparate strains should be induced. Autoimmunity at this level would indicate a profound defect in central tolerance in the chimeras. To identify if the NZB or NZW cells within the chimera are reactive to each other, lymphocytes from both strains of the chimera were tested for proliferation against wildtype NZB, NZW, and/or C57BL/6J strains in a mixed lymphocyte reaction (MLR). When tested against the C57BL/6J cells, both the chimeric NZB and NZW cells proliferated, as expected, since C57BL/6J has a different haplotype than either strain. However, the chimeric NZB or NZW cells did not proliferate against wildtype NZW or NZB cells. This was significantly less than the normal response of wildtype NZW cells versus NZB, and vice versa (Figure 11B). As a result, the different strains in the NZB;NZW chimeras are not reactive to each other due to differences in haplotype.

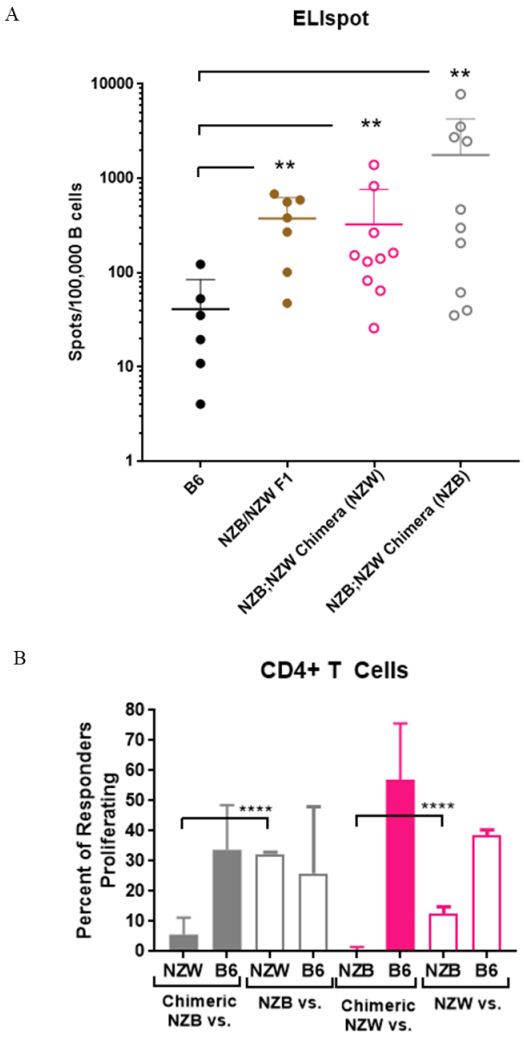


Figure 11. Both NZB and NZW B Cells Produce IgG anti-dsDNA Autoantibodies but are Tolerant to Each Other.

(A) Splenocytes from NZB;NZW chimeras and wildtype strains were sorted based on their MHCI haplotype and tested for IgG anti-dsDNA antibody production via ELIspot. The number of spots 100,000 B cells was calculated. (B) Lymphocytes from NZB;NZW chimeras were co-cultured with lymphocytes from CFSE labelled wildtype NZB, NZW, or B6 mice for 3 days and tested for proliferation using KI-67 staining via FACS. ** = $p \leq 0.01$, **** = $p \leq 0.0001$ using the Mann-Whitney test.

Tolerance Checkpoints

Both breaks in peripheral and central tolerance mechanisms can leads to autoimmunity. As there were no changes identified in Treg cells in the periphery compared to wildtype or non-hematopoietic chimeras, we next investigated the developmental subsets of the lymphocytes in the thymus and bone marrow.

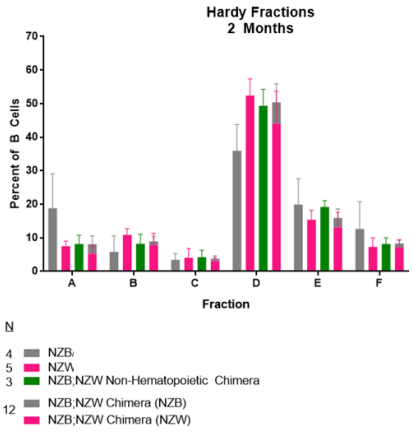
In the bone marrow, there were no major differences in the percentage of the different Hardy fractions or progenitor/stem cell populations compared to control mice (Figure 12A, B). In the progenitor/stem cells, the cell subsets were composed of similar ratios of NZB and NZW between the groups (Figure 12D). However in the Hardy fractions, there was an approximate 50% reduction of the percentage of NZB between fractions A and B (Figure 12C). Since the transition from Hardy fraction A to B does not correspond to a tolerance checkpoint, there are no obvious differences between the abilities of NZB and NZW B cells to pass different checkpoints.

In the thymus, there are also no major differences in the percentages of the different cell populations between the NZB;NZW chimeras and the wildtype strains (Figure 12E). However, there is a large increase of the percentage of NZB cells between the DN and DP thymocyte stages (Figure 12F). During this process, the cells undergo positive selection,

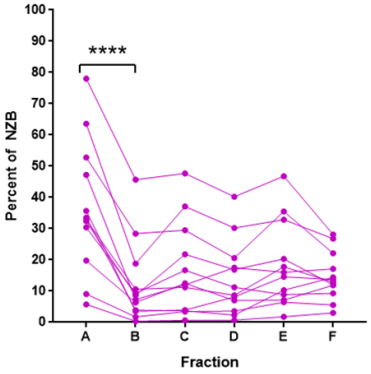
where cells compete for survival based on their responses to MHC/TCR signaling. NZB T cells show greater potential for survival during positive selection than NZW T cells.

Furthermore, when compared to peripheral cell subsets, there were still much greater percentages of NZB T cells and reduced percentages of NZB B cells than could be accounted for based on the percentage of NZB in the progenitor/stem cell populations. This is likely due the changes we see between fraction A and B in the bone marrow, and between DN and DP cells in the thymus (Figure 12G).

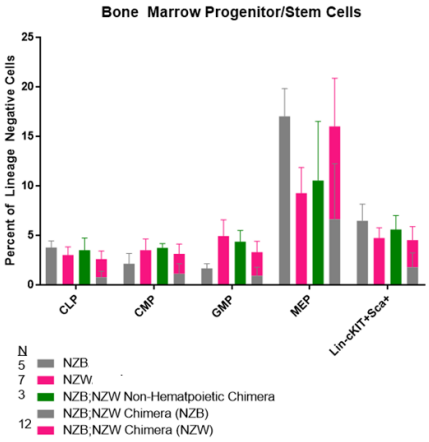
A


Hardy Fractions
2 Months
Percent of B Cells
Fraction
A
B
C
D
E
F
N
4 NZB
5 NZW
3 NZB;NZW Non-Hematopoietic Chimera
12 NZB;NZW Chimera (NZB)
NZB;NZW Chimera (NZW)

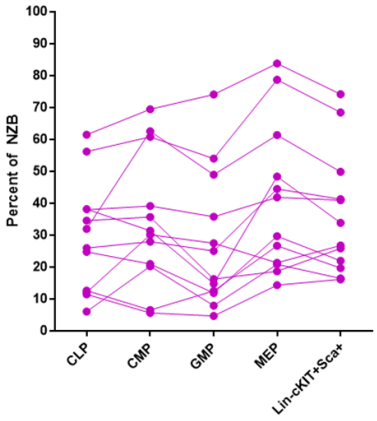
C


****
Percent of NZB
Fraction
A
B
C
D
E
F

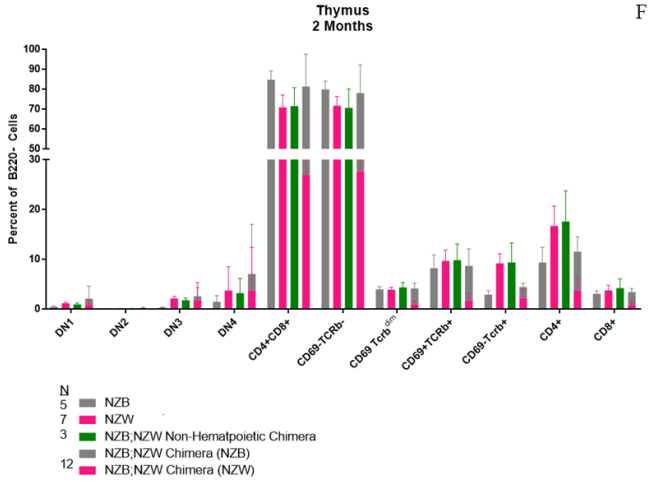
B


Bone Marrow Progenitor/Stem Cells
Percent of Lineage Negative Cells
CLP
CMP
GMP
MEP
Lin-cKIT+Sca+
N
5 NZB
7 NZW
3 NZB;NZW Non-Hematpoietic Chimera
12 NZB;NZW Chimera (NZB)
NZB;NZW Chimera (NZW)

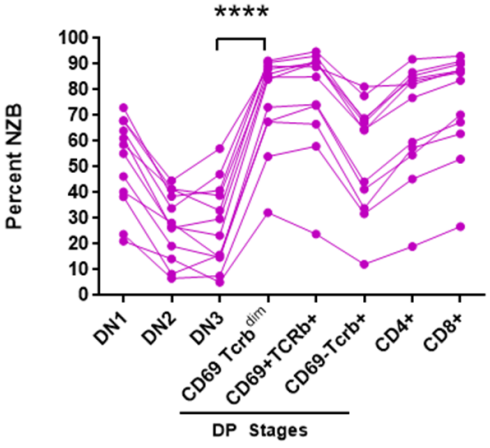
D


Percent of NZB
CLP
CMP
GMP
MEP
Lin-cKIT+Sca+

E


Thymus
2 Months
Percent of B220- Cells
DN1
DN2
DN3
DN4
CD4+CD8+
CD69-TCRb-
CD69 Tcrb dim
CD69+TCRb+
CD69-Tcrb+
CD4+
CD8+
N
5 NZB
7 NZW
3 NZB;NZW Non-Hematpoietic Chimera
12 NZB;NZW Chimera (NZB)
NZB;NZW Chimera (NZW)

F


****
Percent NZB
DN1
DN2
DN3
CD69 Tcrb dim
CD69+TCRb+
CD69-Tcrb+
CD4+
CD8+
DP Stages

G

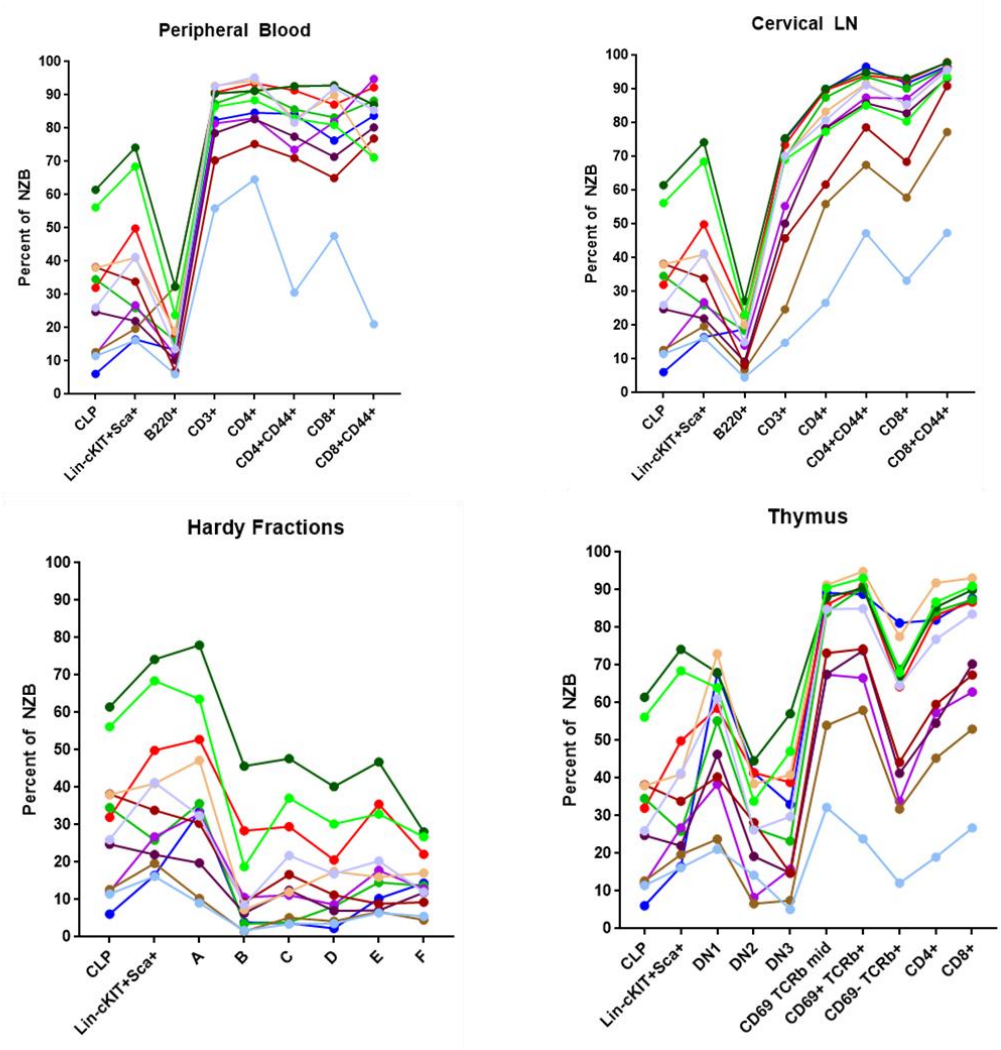


Figure 12. NZB Double Positive T Cells and NZW Bone Marrow B Cells have Enhanced Survival

(A, B, E) Averaged percent of cell populations for wildtype mice and NZB;NZW Chimeras at 2 months of age. Stacked bars represent the combined total of NZB and NZW cells within the chimeras. (C, D, F, G) Individual values of the percent of each cell population that comes from NZB origin. Lines connecting datapoints indicate data are from the same mouse. **** = $p \leq 0.0001$ using a paired T test.

CHAPTER SEVEN
CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Overview

The NZB;NZW chimeric model overcomes many of the obstacles that have prevented the determination of disease etiology in the NZB/NZW F1 or inbred NZM strains. Heterozygosity in the NZB/NZW F1 may mask maladaptive interactions that initiate disease in homozygous cells of the parental strains. Meanwhile, homozygous NZM strains prevent the full investigation into the genetic contribution of each strain, as alleles are lost during inbreeding. By utilizing NZB;NZW chimeras, we show unambiguously that intercellular transactions between these two strains lead to autoimmunity and that NZB T cells specifically cause disease, likely due to increased survival from interactions with NZW MHC during positive selection. Similar transactions may occur in NZB/NZW F1 hybrids as well, but cannot be isolated when all cells in the hybrid are genetically identical. The NZB;NZW chimeras highlight features due to these intercellular interactions, with precocious and increased autoantibody production, severe lymphadenopathy, and splenomegaly. Meanwhile, SLE features driven by other factors can be identified, such as glomerulonephritis. Previously, many studies have linked MHC haplotype to autoimmunity, without a clear indication of its role. However, the NZB;NZW chimeras provide a means to study the mechanism by which MHC may control autoimmunity in the thymus. Only mice where

NZW MHC (H-2z) interact with NZB T cells develop autoimmunity (Figure 13). Further models such as NZB;NZW$^{Foxn1-/-}$ *or* NZB $^{Foxn1-/-}$;NZW chimeras would allow investigation into these important interactions. Finally, by identifying that NZB autoreactive T cells may develop during positive selection, we have uncovered aberrant central tolerance mechanisms that may result in disease onset. These T cell interactions in the thymus and with B cells may be potential targets to prevent flares and lupus progression.

Autoantibody Titers and Renal Disease

The greater titer of autoantibody in NZB;NZW chimeras does not lead to enhanced renal disease or mortality. This suggests that glomerulonephritis may depend upon epistasis of genes from the two strains. This could occur within kidney cells, with aberrant kidney inflammation or repair, or within immune cells that initiate pathology through their effector functions. Alternatively, the degree of glomerulonephritis could be due to the percent of NZB or NZW chimerism in the kidney and their response to inflammation, since this phenotype varies between individual chimeric mice. There are many factors involved in lupus nephritis including specific tissue factors, immune cell effector functions, and the pathogenic nature/cross-reactivity of the autoantibodies (reviewed in [84]). Autoantibodies may cause tissue damage by directly binding to kidney antigens[64, 187, 321] or binding to apoptotic nucleosomes found in the glomeruli[135, 136, 191]. Once bound, these autoantibodies can induce damage through complement fixation and FCγ receptor signaling. Furthermore, the response

of the kidney to inflammation is important. Indeed in the NZB/NZW F1 model, autoantibody production (*Sle1*) and glomerulonephritis (*Sle3*) can be genetically separated. Part of the genetic influence of the *Sle3* locus is considered to be due to specific alleles of the *Klk* genes, which encode serine proteases that regulate inflammatory pathways in tissues [171, 194]. Without these aberrant inflammatory pathways, antibodies from *Sle1* alone do not cause kidney disease[193]. As a result, NZB;NZW chimeras with greater NZW composition in the kidneys may have enhanced renal pathology due to the NZW alleles of *Klk* genes.

T Cells in Autoimmunity

Within NZB;NZW chimeras, NZB and NZW parental cell types make disproportionate contributions to immune cell subsets. NZB cells preferentially contribute to the T lymphocyte compartment, and especially to the population of CD44+ T cells, while NZW cells preferentially contribute to the B lymphocyte compartment. It is clear that both NZB and NZW B cells are sources of IgG anti-dsDNA antibodies. As such, it appears that the "decision" to break tolerance is made by a different cellular compartment.

T cells have been implicated in SLE pathogenesis in both humans and mice by providing help to autoreactive B cells as well as through their effector functions. In the parent-into-F1 model of SLE, T cells are transferred from the parental strain into the F1 hybrid offspring. In this model, the transferred CD4+ T cells drive autoreactivity in both the recipient and co-

transferred B cells, leading to SLE-like disease [75]. Additionally, recent work has shown that inhibiting CD4+ T cell metabolism in C57BL/6J containing *Sle1*, *Sle2*, and *Sle3*, reverses the SLE phenotype in aged mice [315, 316].

After specifically removing NZB T cells from the NZB;NZW chimeras, it was shown that these cells are required for strong, penetrant disease. These mice do not develop the lymphadenopathy, splenomegaly, or renal pathology seen in the NZB;NZW chimeras. As a result, we infer that NZB T cells are chiefly required for autoimmunity. In the thymi of NZB;NZW chimeric mice, NZB T cells have a competitive advantage during positive selection, such that the majority (~80%) of T cells that egress from the thymus are of NZB origin. It would appear that NZB T cells are more capable of survival during positive selection in the chimeric thymus environment. Although NZB T cells are necessary for substantial disease, they alone are not sufficient, as wildtype NZB mice do not develop SLE features. We hypothesize that NZW thymic epithelial cells may be unique in their ability to drive the survival of NZB T cells during positive selection, insofar as NZB;C57BL/6J chimeras do not display an exaggerated NZB T cell compartment as NZB;NZW chimeras do.

During positive selection, T cells first interact with MHC molecules and respond to survival signals based on TCR signaling. Perhaps NZB T cells respond positively to NZW thymic cells expressing NZW MHC compared to the NZB strain and through this interaction they receive anti-apoptotic signals that promote their survival out into the periphery.

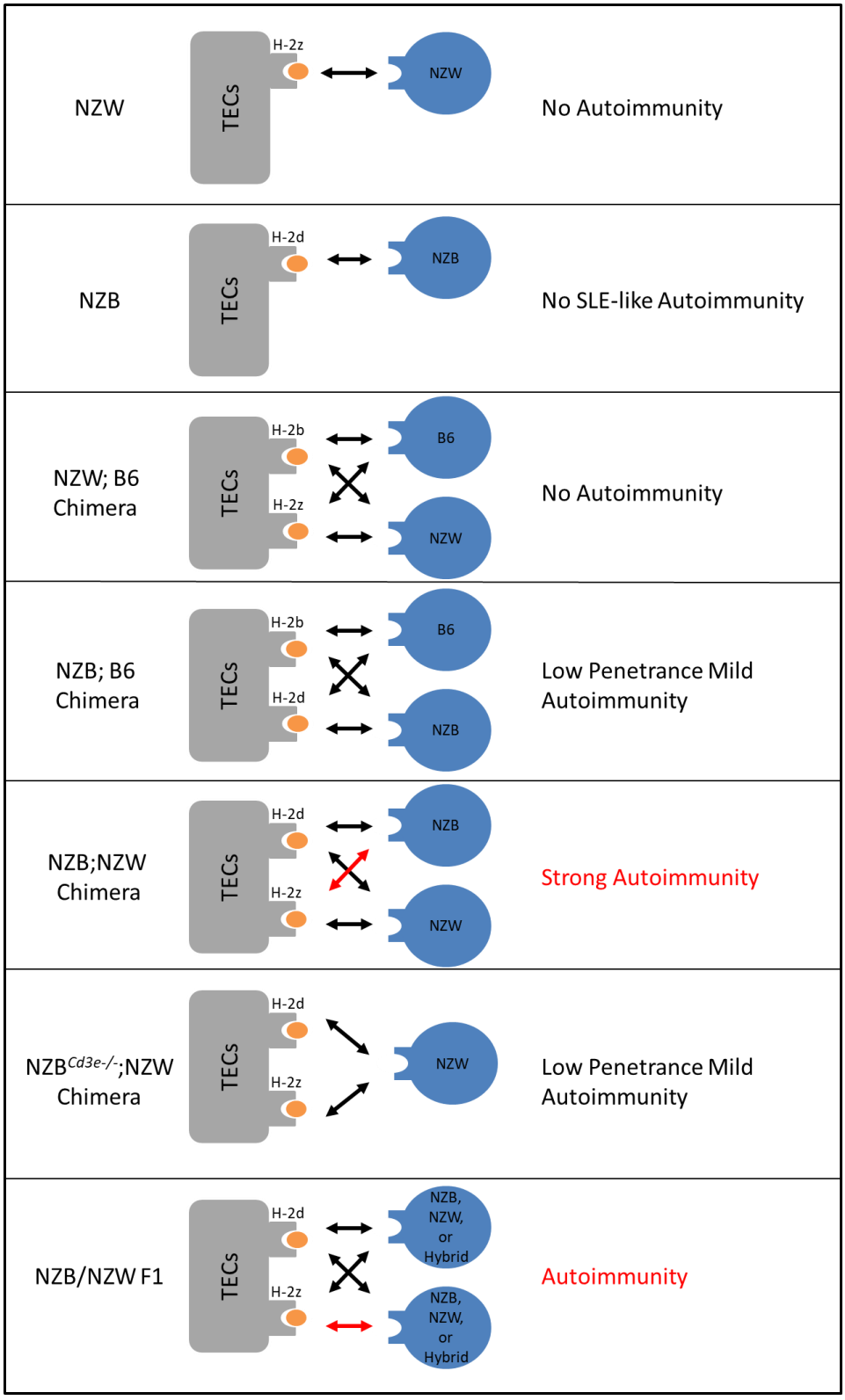
Secondary Causes of Autoimmunity

However 14% (4/29) of the NZB$^{Cd3e-/-}$; NZW chimeras and 22% (10/45) of the NZB;NZW non-hematopoietic chimeras studied developed moderate levels of autoantibodies. In both of these chimeras all the T cells are derived from the NZW parental strain. Although these mice can develop IgG anti-dsDNA, this occurs approximately 2-3 months later than NZB;NZW chimeras and these mice do not develop splenomegaly or lymphadenopathy. Due to the differences in phenotype, it is possible there is a secondary etiology of disease in these mice that occurs less frequently than the fully penetrant phenotype of the NZB;NZW chimeras. Since this occurs in the absence of the NZB hematopoietic lineage, the interaction between NZB extra-hematopoietic cells and NZW must initiate disease. The mice are housed in the same specific-pathogen-free strictly maintained environment and the mice that develop autoimmunity are not necessarily cage- or litter-mates, so it is likely not due to environmental differences such as light exposure, microbiota, or pathogen infection. However, one possible etiology could be the expression of endogenous viruses found in the two strains. High titers of circulating gp70 are found in NZB/NZW F1 and NZB mice. Furthermore anti-gp70 antibodies are produced in SLE mouse models and are observed in immune complexes in the kidneys[126, 185]. It has been hypothesized that expression of viral transcripts and virions from these endogenous retroviruses promote autoimmunity by stimulating TLR7 and acting as an antigenic source[20]. Since NZB mice are known to express

high titers of endogenous retroviruses, it is possible that in NZB;NZW chimeras this could lead to a response by NZW immune cells. This would not require the presence of NZB hematopoietic lineage cells, as gp70 is produced by hepatocytes similar to acute phase proteins[107]. As a result, in the absence of autoreactive NZB T cells that drive disease, autoantibodies may be produced in response to these viral transcripts but be limited to low penetrance autoimmunity.

Figure 13. Interactions of thymic epithelial cells and developing DP T cells in wildtype strains and chimeras.

In all the mice strains studied (NZB;B6, NZW;B6, NZB;NZW, NZB$^{Cd3e-/-}$;NZW, NZB, NZW, and NZB/NZW F1), only strains where NZB cells interact with H-2z thymic epithelial cells (TECs) develop strong autoimmunity (arrows shown in red). In NZB/NZW F1 mice, these interactions likely occur at lower frequencies. T cells do not undergo allelic exclusion, so the resulting TCR can result from a combination of NZB and NZW TCR alleles. On the other hand, in NZB;NZW chimeras, TCRs are derived completely from NZB and NZW alleles in the homozygous cells. This corresponds with the increase of autoimmunity seen in the NZB;NZW chimeras, compared to NZB;NZW F1.


NZW
TECs
H-2z
NZW
No Autoimmunity
NZB
TECs
H-2d
NZB
No SLE-like Autoimmunity
NZW; B6 Chimera
TECs
H-2b
H-2z
B6
NZW
No Autoimmunity
NZB; B6 Chimera
TECs
H-2b
H-2d
B6
NZB
Low Penetrance Mild Autoimmunity
NZB;NZW Chimera
TECs
H-2d
H-2z
NZB
NZW
Strong Autoimmunity
NZB$^{Cd3e-/-}$;NZW Chimera
TECs
H-2d
H-2z
NZW
Low Penetrance Mild Autoimmunity
NZB/NZW F1
TECs
H-2d
H-2z
NZB, NZW, or Hybrid
NZB, NZW, or Hybrid
Autoimmunity

RECOMMENDATIONS

This study has shown that NZB;NZW embryonic chimeras develop severe autoimmunity earlier than NZB/NZW F1 hybrids and that this is mediated by NZB T cells. This elucidates intercellular pathogenic mechanisms necessary for autoimmune disease between these strains previously masked in the NZB/NZW F1 model and implicates T cells in the etiology of disease. However, there are still many questions that require investigation.

NZB T Cells in Autoimmunity

To confirm that NZB T cells are able to induce autoimmunity, T cell transfer experiments can be performed. NZB T cells from NZB;NZW chimeras can be collected and transferred into NZB$^{Cd3e-/-}$;NZW chimeras to see if the SLE phenotype can be restored. However, it is also possible that NZB and NZW T cells, in conjunction, potentiate disease. As a result, generating NZB;NZW$^{Cd3e-/-}$ chimeras will identify if NZB T cells alone, or both NZB and NZW T cells, are required.

Through these studies we have identified that NZB T cells can drive disease, and this may be due to the enhanced survival of DP T cells during positive selection. The signals that developing DP thymocytes require to survive are from interactions with the MHC on cTECs in the thymus. These signals are regulated by the TCR genes of the mouse strain, the

downstream proteins that modulate the signal sensitivity and strength, the peptides presented on the MHC, as well as the anti-apoptotic pathways in the T cell. These factors may differ in NZB T cells in comparison to NZW, which promote their survival. For example, NZB TCR may inherently have enhanced binding ability to the H-2z haplotype. This could be assessed *in vitro* by culturing early thymic progenitors of NZB and NZW in thymic tissues of various haplotypes and identifying the cell populations and calcium flux that occurs as they go through selection. In addition to survival through positive selection, NZB autoreactive T cells must also be able to survive through negative selection. Negative selection occurs through strong stimulation of the TCR, which results in apoptosis. NZB T cells may survive better than NZW during positive and negative selection due to increased expression of the anti-apoptotic molecule BCL-2. NZB mice, but not NZW, have a mutation in *miR16*, leading to reduced expression of this miRNA that aids in the degradation of *BCL-2* transcripts. As a result, this increase in BCL-2 is thought to cause the CLL in NZB mice [237]. However, this increase may also be important in the survival of NZB T cells during central tolerance. This could be assayed by monitoring BCL-2 levels in the NZB and NZW T cells in the thymic populations of the NZB;NZW chimeric mice.

NZW in Autoimmunity

NZB cells drive the SLE phenotype in NZB;NZW chimeras, but not NZB;C57BL/6 or a wildtype homozygous strain. This indicates that the NZB and NZW combination promotes

autoimmunity through intercellular transactions. However, the contribution of NZW is undetermined. Since the NZB T cell phenotype begins in the thymus with an increase of NZB DP T cells, it is possible NZW is acting in the thymus to promote this expansion.

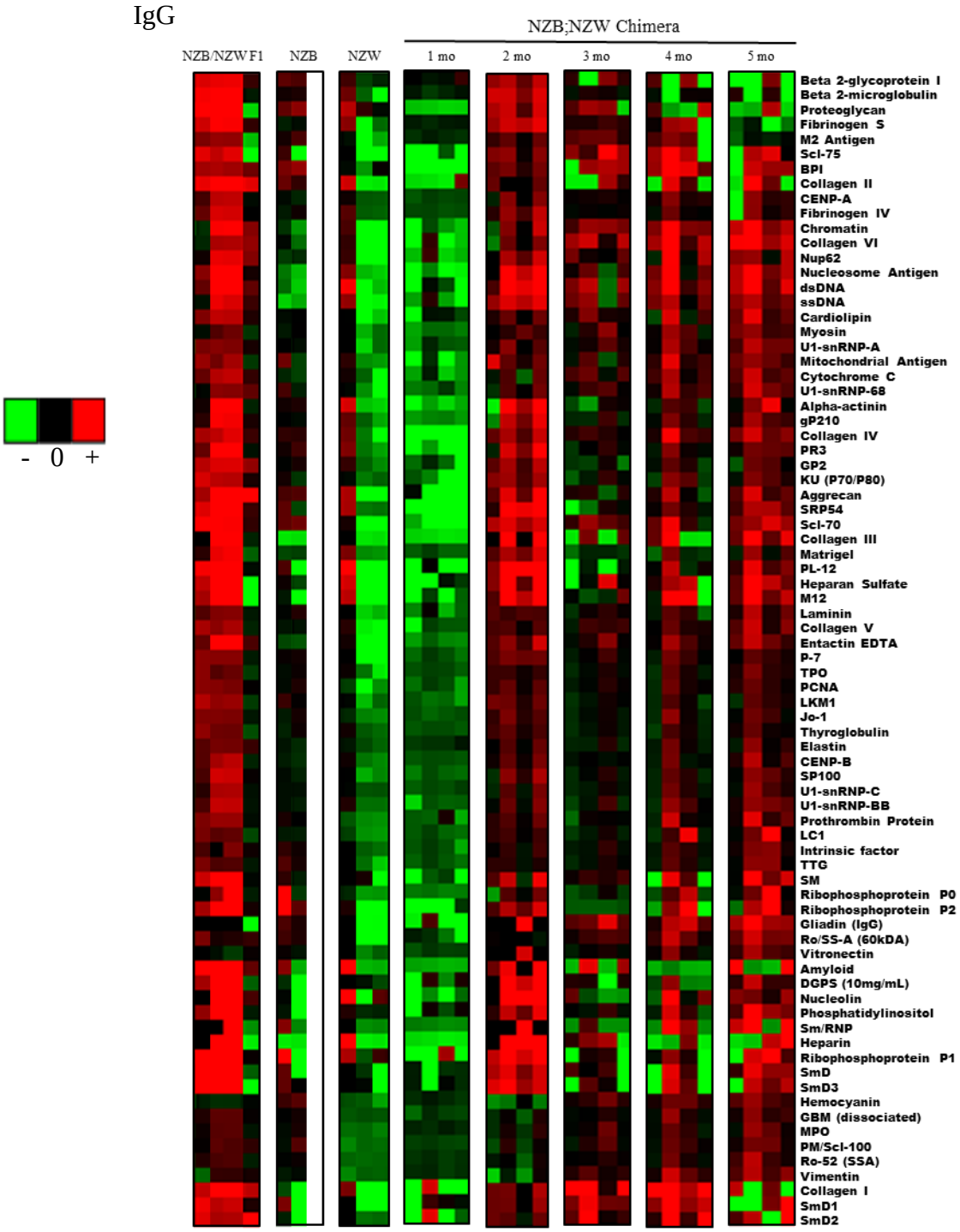
Initially this could be characterized by identifying the percentage of NZB and NZW cTECS in the thymus via FACS or immunohistochemistry and correlating this to the NZB T cell expansion or autoimmunity[130].

Then, the effect of the NZW thymic tissues in the chimeras could be assessed by three different methods. First, NZB;NZW chimeras could be developed lacking thymic tissue from one strain or the other, through NZB$^{Foxn1-/-}$;NZW and NZB;NZW$^{Foxn1-/-}$ chimeras. The hypothesis is that the former would develop autoimmunity while the latter would not. Meanwhile, a chimera with NZB$^{Cd3e-/-}$; NZW$^{Foxn1-/-}$ would develop no disease, while a NZB$^{Foxn1-/}$; NZW$^{Cd3e-/-}$ would develop particularly severe disease (with all T cells of NZB origin being selected on thymic epithelium of NZW origin). Another method would be to perform a thymic transplant by irradiating a wildtype parental strain neonate and transplanting the thymus into a thymectomized NZB;NZW chimera neonate[235]. In both of these models the frequency of the thymocytes can be identified to discover if there are any changes when selected on different thymic tissue. Furthermore, changes in the SLE phenotype can be observed. In addition to these *in vivo* experiments, *in vitro* thymic culture provides a rapid method to assay T cell responses in various combinations of thymic

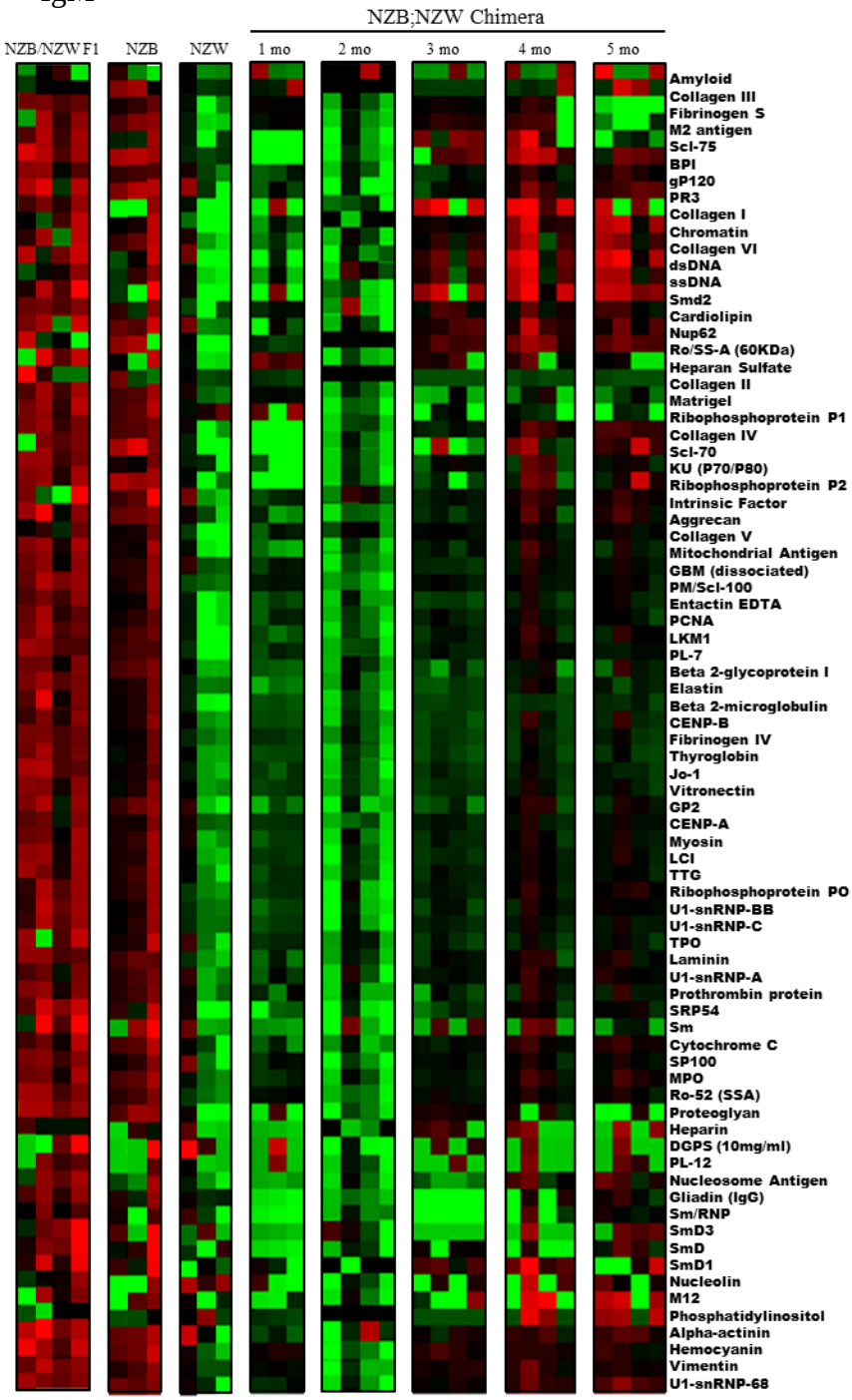
tissue[214]. Testing NZB, NZW, or a competitive test of each in combination with NZB, NZW, and B6 thymi would be especially informative to see if these changes occur in the wildtype state or only occur within certain chimeric combinations. Ultimately if differences are found, changes in signaling pathways or downstream effector molecules could be distinguished by RNAseq and western blot. For example, changes in mTOR signaling, glucose metabolism, and TCR signaling, as alternations in these pathways have been identified[315].

Finally, the mechanism by which NZW MHC could induce NZB T cell survival may be due to differences in the NZW haplotype gene sequence, expression, or the peptides presented. This could be assessed by eluting the peptides from the MHC of cTECS and identifying differences between haplotypes, or looking at the sequences and expression levels of the MHC components, which have been shown to influence DP T cell responses[199].

APPENDIX A
COMPLETE PROTEIN MICROARRAY HEAT MAP OF IGG AND IGM BINDING SPECIFICITIES OF NZB;NZW CHIMERAS

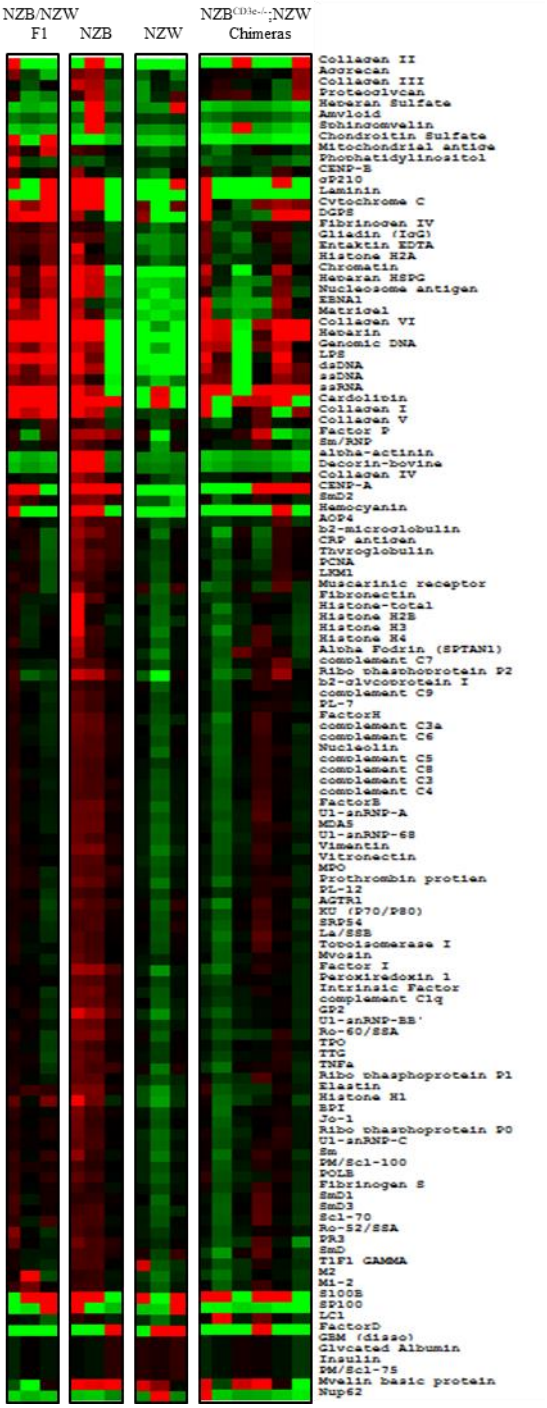


IgM


NZB;NZW Chimera
NZB/NZW F1
NZB
NZW
1 mo
2 mo
3 mo
4 mo
5 mo
Amyloid
Collagen III
Fibrinogen S
M2 antigen
Scl-75
BPI
gP120
PR3
Collagen I
Chromatin
Collagen VI
dsDNA
ssDNA
Smd2
Cardiolipin
Nup62
Ro/SS-A (60KDa)
Heparan Sulfate
Collagen II
Matrigel
Ribophosphoprotein P1
Collagen IV
Scl-70
KU (P70/P80)
Ribophosphoprotein P2
Intrinsic Factor
Aggrecan
Collagen V
Mitochondrial Antigen
GBM (dissociated)
PM/Scl-100
Entactin EDTA
PCNA
LKM1
PL-7
Beta 2-glycoprotein I
Elastin
Beta 2-microglobulin
CENP-B
Fibrinogen IV
Thyroglobin
Jo-1
Vitronectin
GP2
CENP-A
Myosin
LCI
TTG
Ribophosphoprotein PO
U1-snRNP-BB
U1-snRNP-C
TPO
Laminin
U1-snRNP-A
Prothrombin protein
SRP54
Sm
Cytochrome C
SP100
MPO
Ro-52 (SSA)
Proteoglyan
Heparin
DGPS (10mg/ml)
PL-12
Nucleosome Antigen
Gliadin (IgG)
Sm/RNP
SmD3
SmD
SmD1
Nucleolin
M12
Phosphatidylinositol
Alpha-actinin
Hemocyanin
Vimentin
U1-snRNP-68

APPENDIX B
COMPLETE PROTEIN MICROARRAY HEAT MAP OF IGG AND IGM BINDING SPECIFICITIES OF NZB$^{CD3E-/-}$;NZW CHIMERAS

IgG

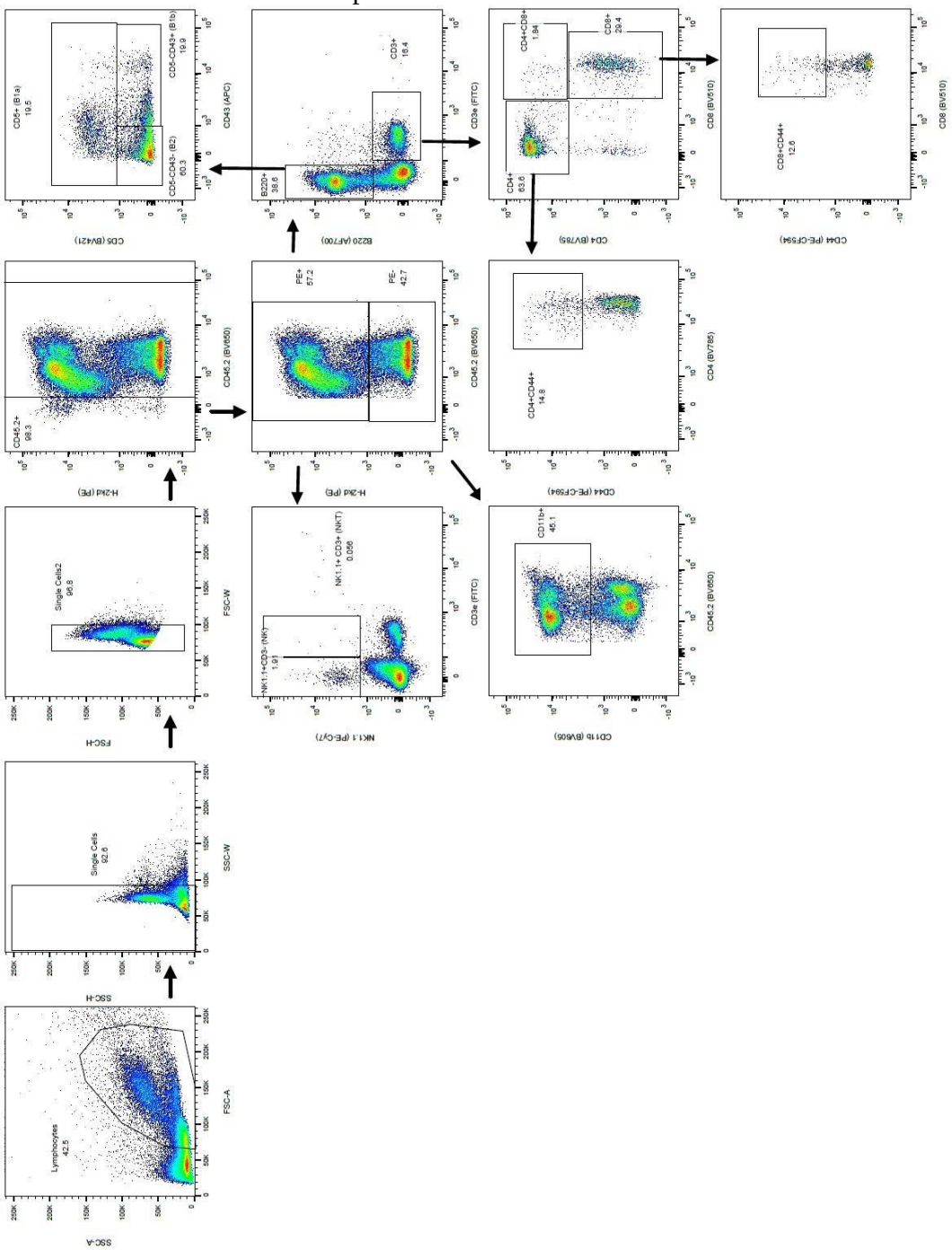


IgM

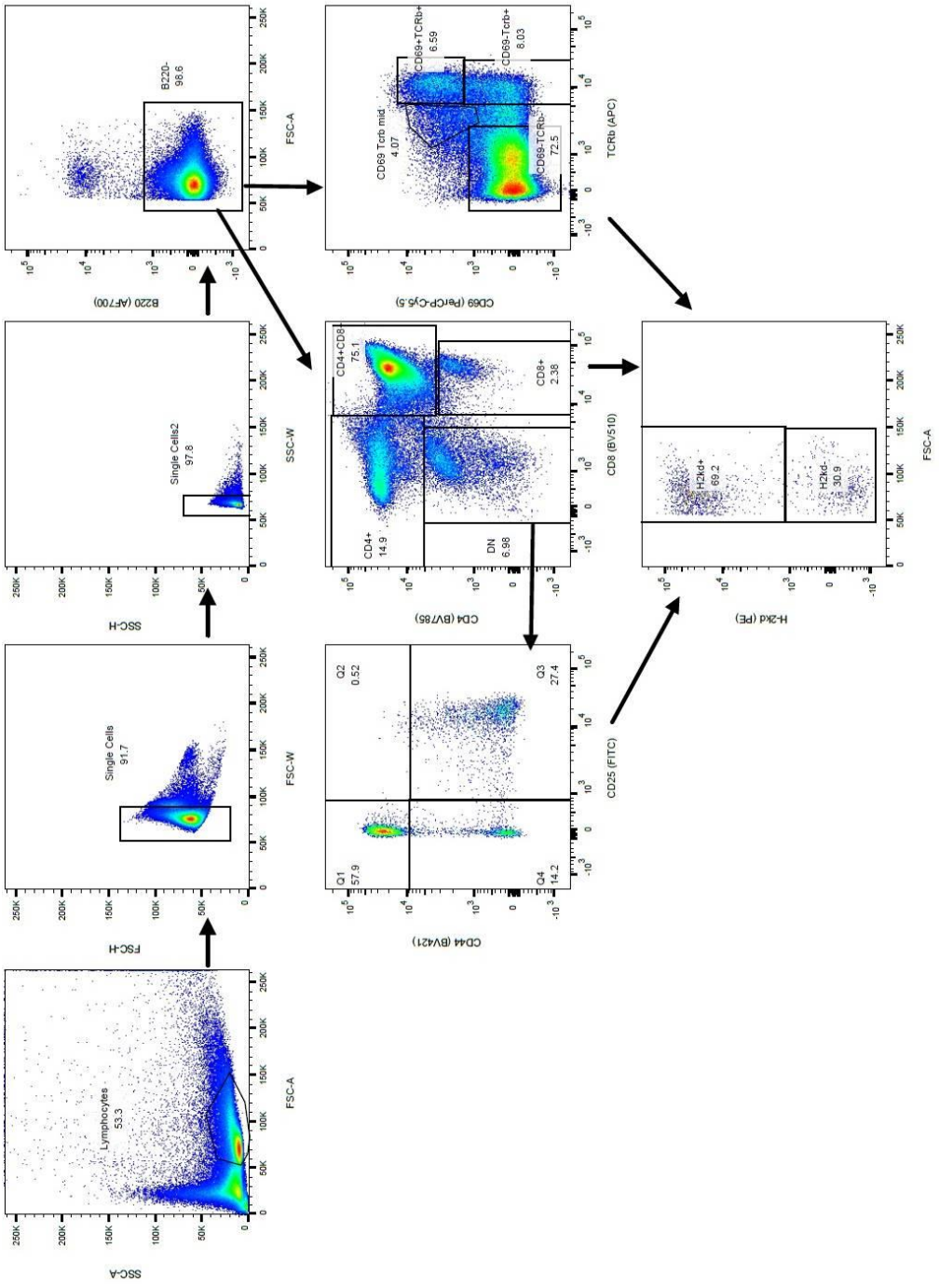

NZB/NZW F1
NZB
NZW
NZB CD3e-/-;NZW Chimeras
Collagen V
DGP8
Insulin
Sphingomyelin
FactorD
Cytochrome C
Glycated Albumin
Collagen III
Myelin basic protein
Cardiolipin
Alpha Fodrin (SPTAN1)
SP100
Collagen II
Heparin
EBNA1
dsDNA
ssDNA
LPS
Amyloid
Decorin-bovine
alpha-actinin
Factor P
Heparan Sulfate
PM/Scl-75
Chondroitin Sulfate
Genomic DNA
Laminin
Collagen VI
Hemocyanin
Chromatin
Nucleosome antigen
b2-microglobulin
CRP antigen
Collagen IV
Aggrecan
AQP4
PCNA
Elastin
GP2
Thyroglobulin
Ro-60/SSA
Proteoglycan
Collagen I
Factor I
FactorH
complement C1q
Entaktin EDTA
CENP-B
complement C6
complement C7
complement C8
Fibronectin
complement C4
BPI
complement C5
LC1
complement C3
complement C9
Myosin
PL-12
PL-7
Jo-1
TTG
Prothrombin protien
TPO
Gliadin (IgG)
Heparan HSPG
Mitochondrial antige
FactorB
Peroxiredoxin 1
Matrigel
Intrinsic Factor
Vimentin
Vitronectin
MDA5
Ribo phasphoprotein P0
LKM1
M2
Muscarinic receptor
Ribo phasphoprotein P2
PR3
CENP-A
GBM (disso)
gP210
SmD2
Fibrinogen IV
b2-glycoprotein I
TNFa
Fibrinogen S
complement C3a
AGTR1
Sm/RNP
Histone H1
La/SSB
Histone H2B
POLB
Histone H2A
Mi-2
KU (P70/P80)
Topoisomerase I
Scl-70
PM/Scl-100
Ro-52/SSA
U1-snRNP-68
U1-snRNP-A
MPO
Ribo phasphoprotein P1
T1F1 GAMMA
U1-snRNP-C
Histone-total
Histone H3
Histone H4
SRP54
SmD1
SmD3
Sm
U1-snRNP-BB'
S100B
Nucleolin
SmD
Phosphatidylinositol
ssRNA
Nup62

APPENDIX C
FLOW CYTOMETRY GATING STRATEGIES

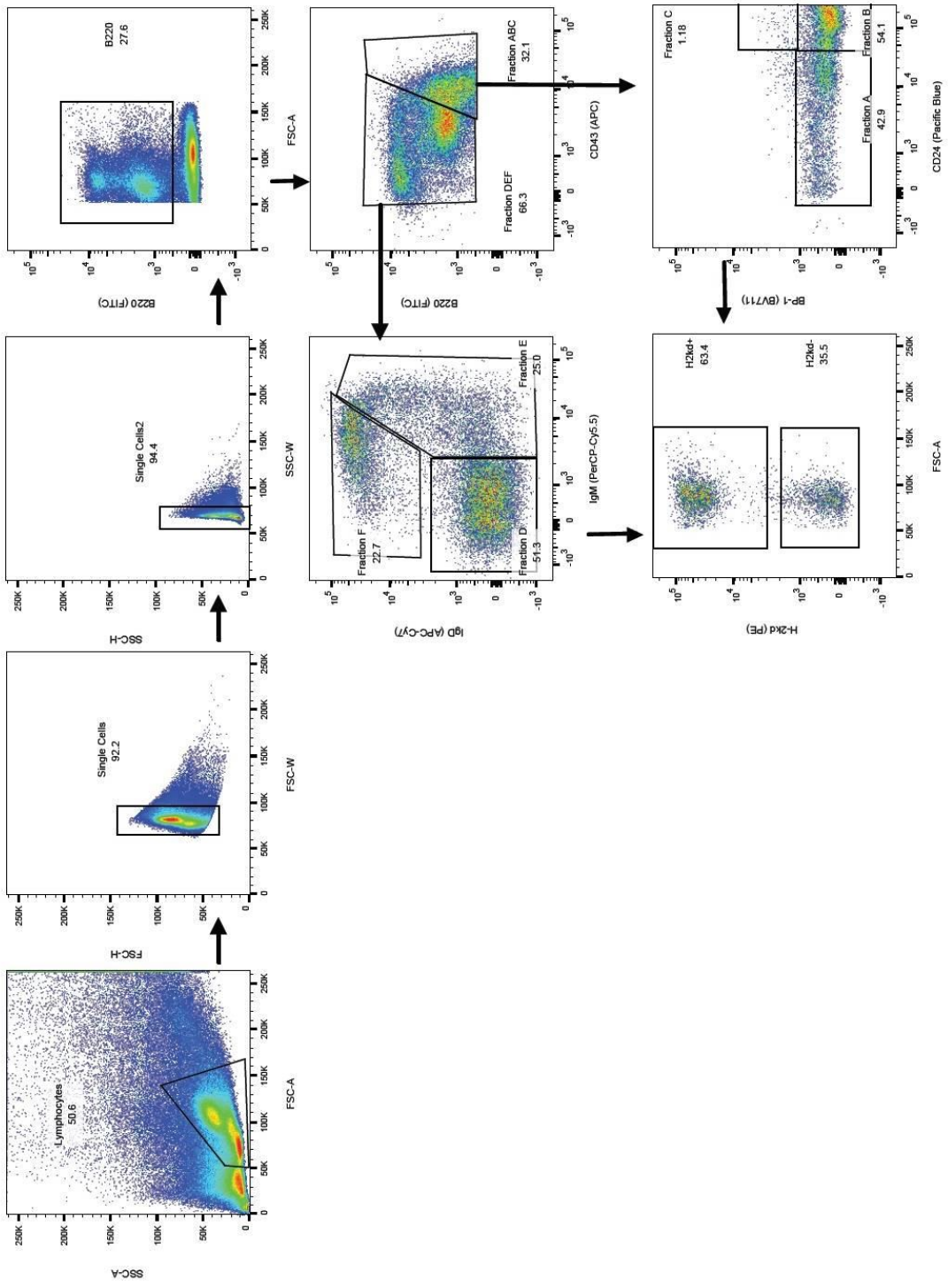
Peripheral Immune Cell Panel



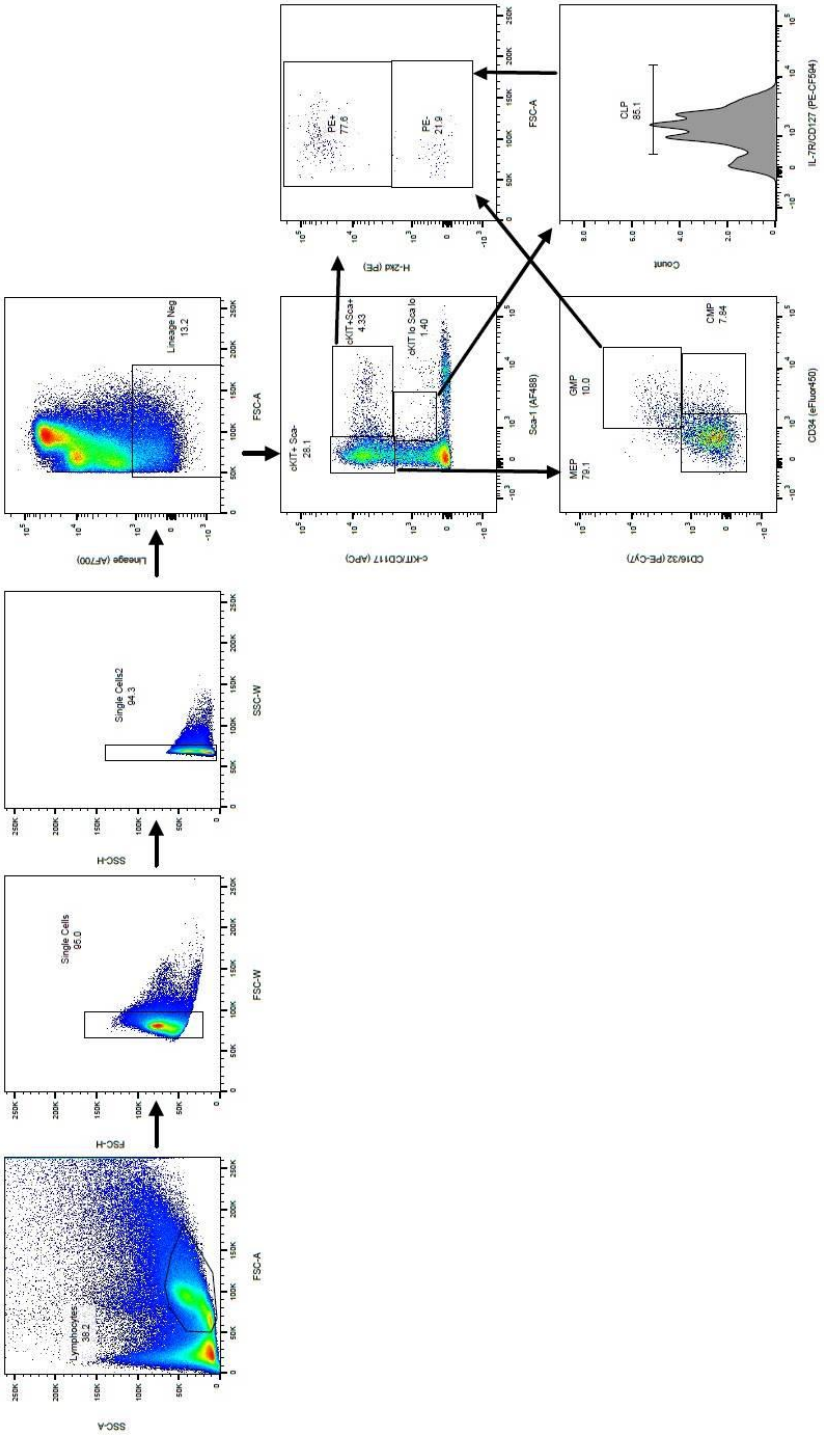
Thymus Panel



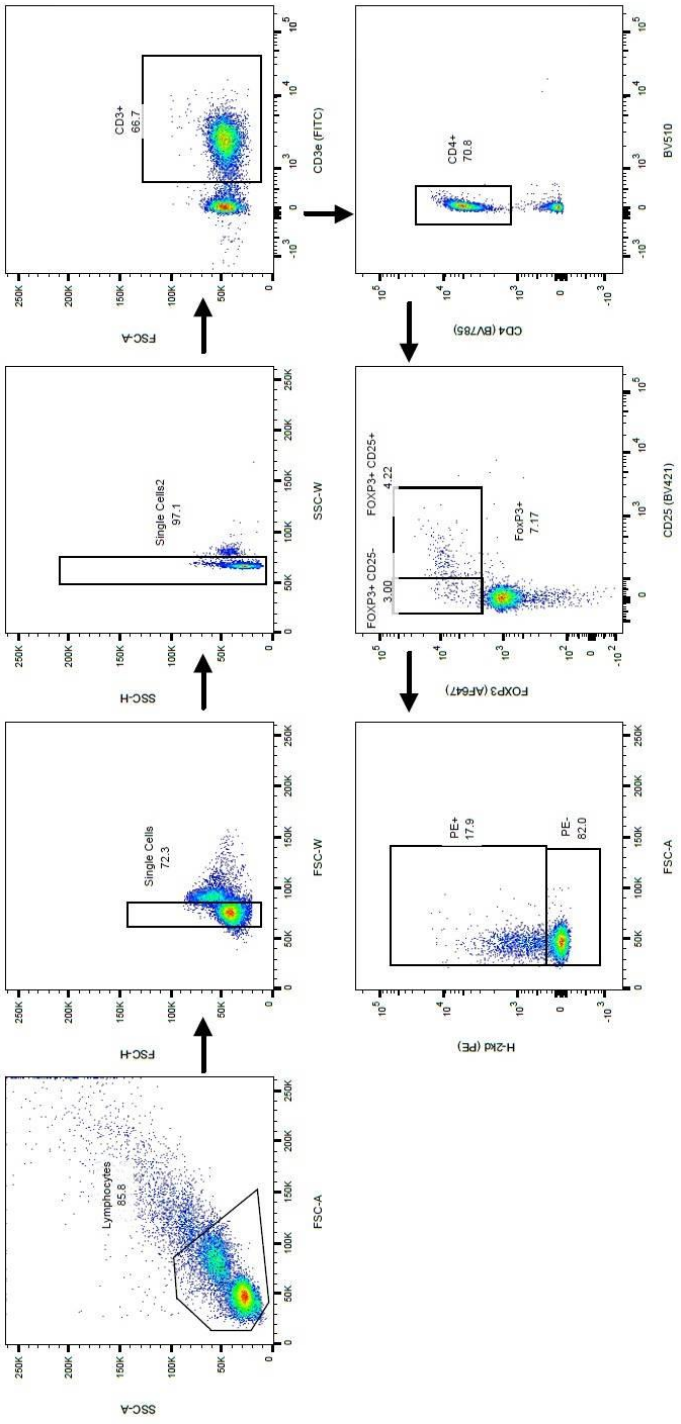
Hardy Fractions Panel



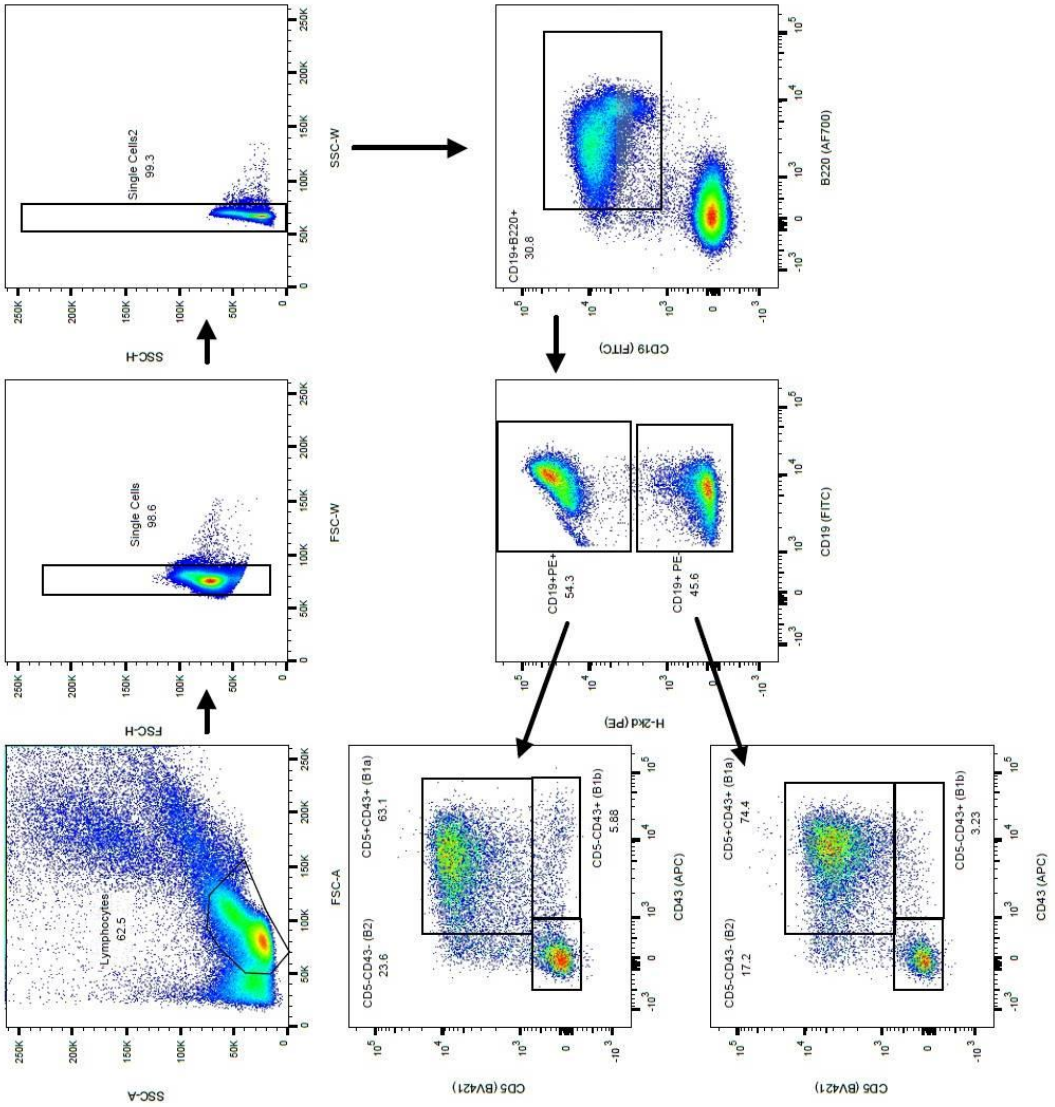
Hematopoietic Stem Cell Panel



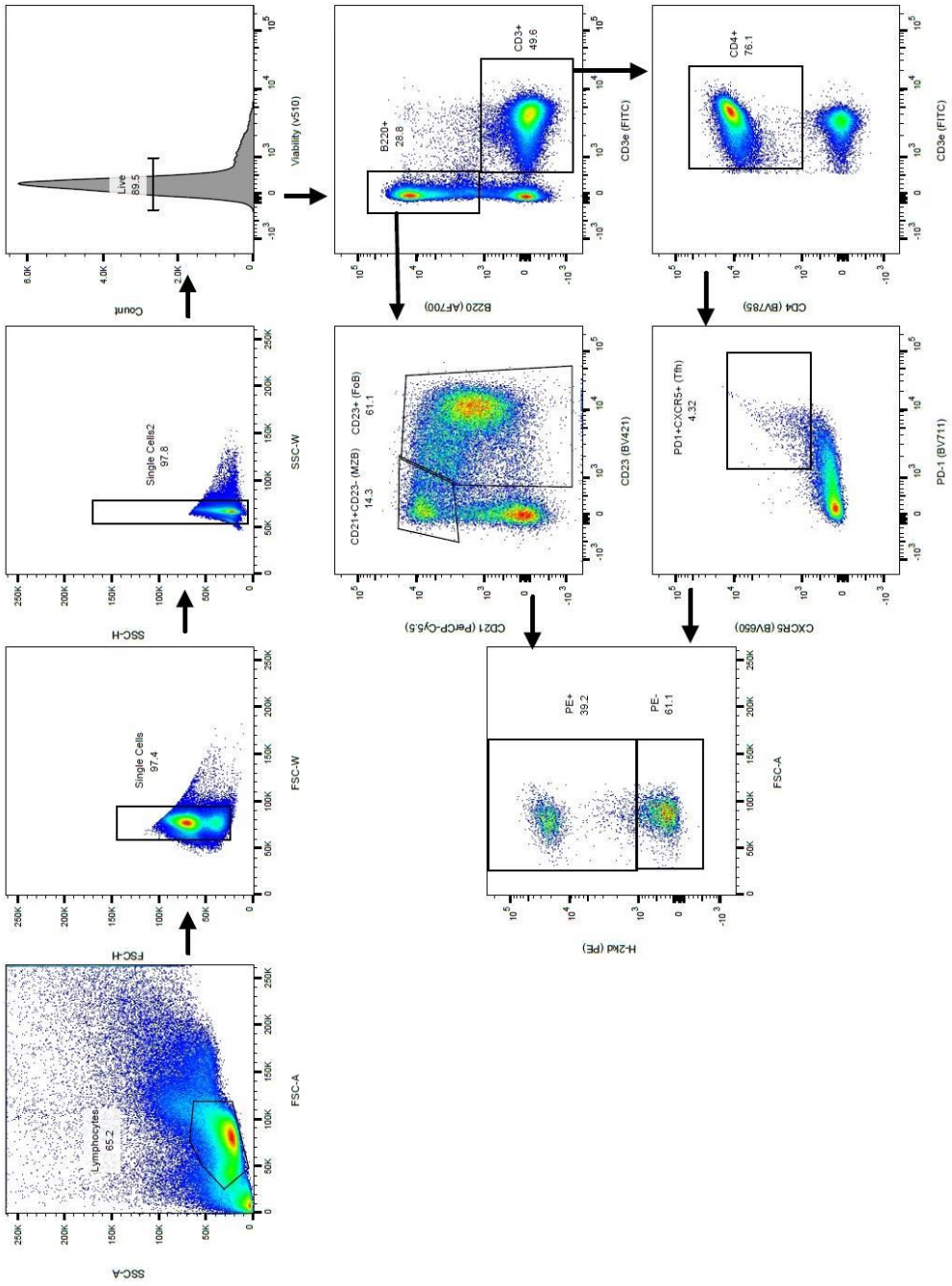
FOXP3 Panel


SSC-A
FSC-A
Lymphocytes
85.8
FSC-H
FSC-W
Single Cells
72.3
SSC-H
SSC-W
Single Cells2
97.1
FSC-A
CD3e (FITC)
CD3+
66.7
CD4 (BV785)
BV510
CD4+
70.8
FOXP3 (AF647)
CD25 (BV421)
FOXP3+ CD25-
3.00
FOXP3+ CD25+
4.22
FoxP3+
7.17
H-2k4 (PE)
FSC-A
PE+
17.9
PE-
82.0

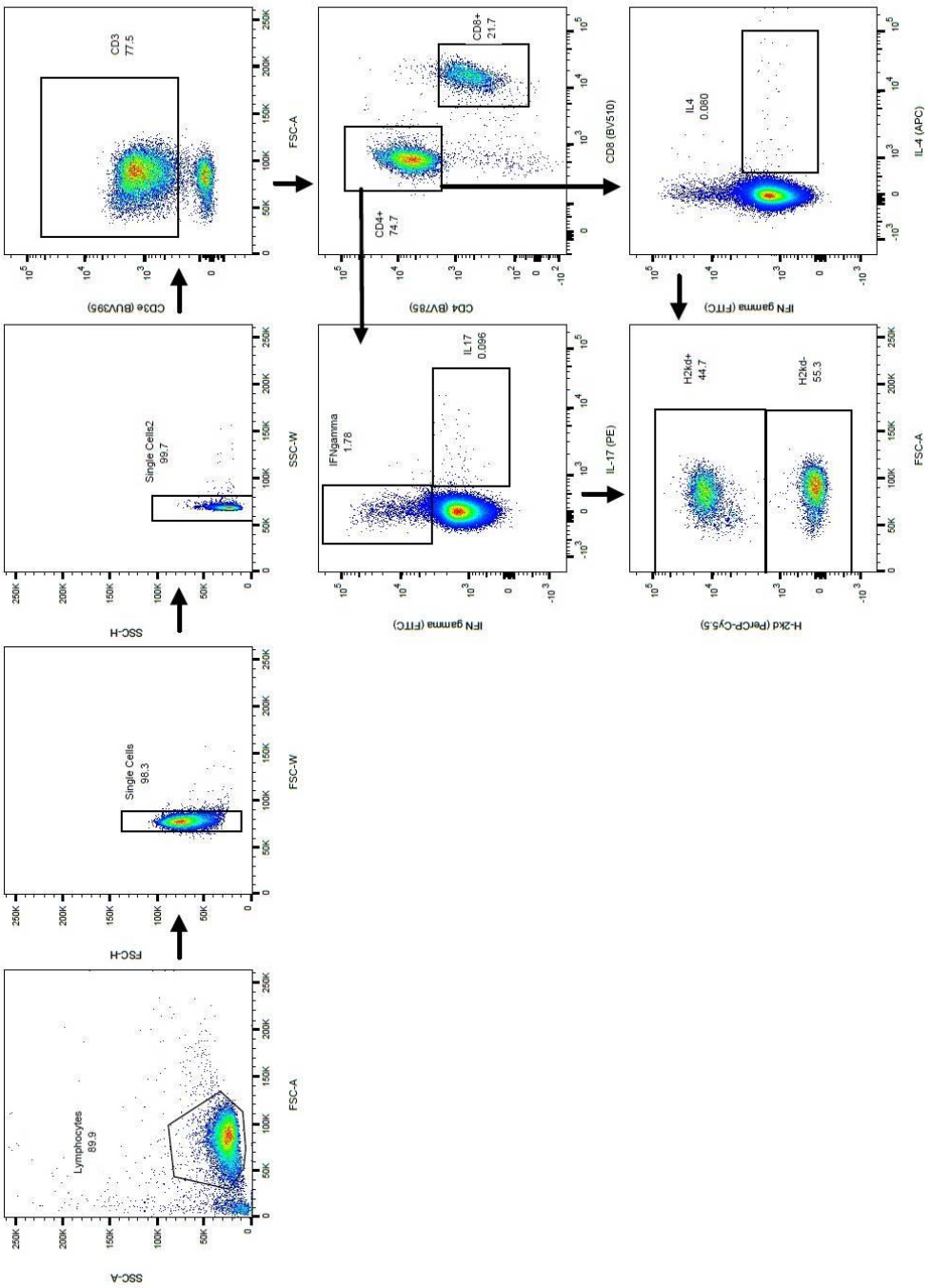
Peritoneal Panel



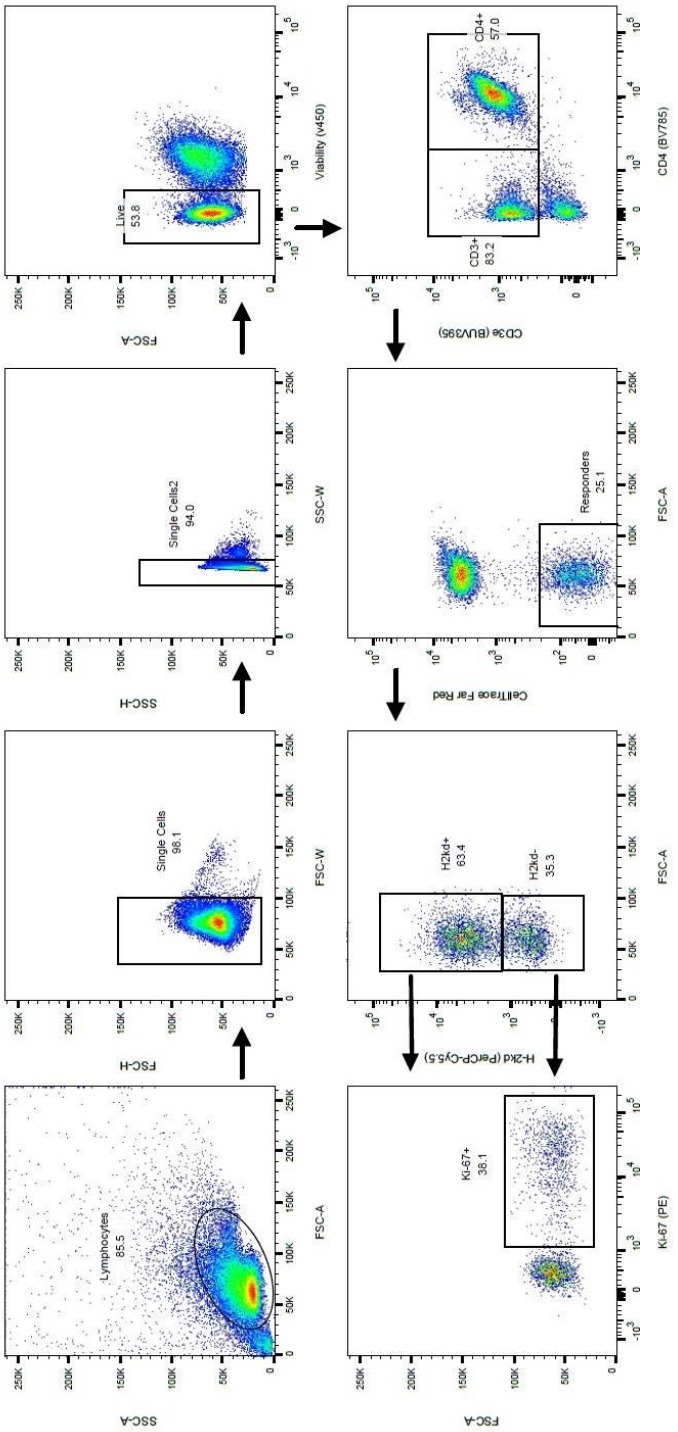
Follicle Panel



T Cell Subsets Panel



Ki-67 Proliferation Panel



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