THE CONTRIBUTION OF IFNα-STIMULATED IMMUNE CELL POPULATIONS TO B6.NBA2 LUPUS-LIKE DISEASE

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Contents

List of Tables 8

List of Figures 9

Acknowledgements 10

Abstract 11

CHAPTER 1: Background 12

- What is SLE? 12
- Sex-related differences in SLE 12
- Animal models of SLE 13
  - NZWxNZB F1 (BW) 13
  - NZM 14
  - B6.Nba2 14
  - B6.Sle1 16
  - Murphy Roths Large/ lymphopoliferation (MRL/lpr) 16
  - Pristane Induced Model 17
  - BXSB 17
- Involved cell subtypes 18
  - Plasmacytoid dendritic cells (pDCs) 18
  - B cells 19
  - T cells 21
  - Myeloid cells 26
- Cytokines involved in SLE pathogenesis 30
  - IFNα 30
  - IFNγ 32
  - IL-6 33
  - IL-10 33
- Current treatment options for SLE 34
  - General immunosuppression 34
  - Anti-malarials/hydroxychloroquine 35
  - Anti-BAFF antibodies 35
  - Anti-IFNAR antibodies 36
  - Anti-CD20 antibodies 36
- Study Plan 37

Rationale 37
Hypothesis 37
Cell specific IFNAR knockout mice 38
CHAPTER 2: Methods 38
Study Animals 38
Harvest and Processing of Tissues 39
Red Blood Cell (RBCs) Removal 40
Immunohistochemistry 40
Immunofluorescent staining 41
Periodic Acid Schiff (PAS) 41
CD4 & CD8 Staining 42
Imaging 43
Scoring 43
Flow Cytometry 43
Flow Sort 45
Enzyme Linked Immunosorbent Assay (ELISA) 47
In-house ELISAs; Immunoglobulin and Chromatin 47
Manufacturer Kit ELISAs; 48
Immunizations 48
NP-CGG_{21} Immunization 48
NP-Ficoll Immunization 48
ELISpot 49
Plasma Cells IgG Production 49
U-PLEX Mouse Biomarker Assay 49
Quantitative polymerase chain reaction (qPCR) 50
Ex Vivo Experiments 50
Calcium Flux Assay 50
T Helper Cell Skewing 51
Statistics 52
CHAPTER 3: BΔIFNAR 52
Hypothesis and rationale 52
Results: Disease Phenotype 53
B cells in BΔIFNAR mice do not respond to IFNα 54

Splenomegaly is significantly decreased in 4 month old B6.Nba2.BΔIFNAR mice 56
Activation of B cells is significantly decreased in B6.Nba2.BΔIFNAR mice 56
B6.Nba2.BΔIFNAR mice have reduced populations of splenic GC B cells 58
B6.Nba2.BΔIFNAR mice have decreased PC and Memory B Cell populations 59
Reduction of GC B cells in B6.Nba2.BΔIFNAR mice is not accompanied by 60
decreased TFH cells 60
GC B cells from B6.Nba2.BΔIFNAR mice have decreased expression of anti-apoptotic factors 61
B6.Nba2.BΔIFNAR mice have reduced serum ANAs, particularly IgG_{2C} ANAs 62
B6.Nba2.BΔIFNAR mice are not protected from lupus-like glomerular disease 63
Type I IFN stimulated B cells have a larger pathogenic contribution early in disease 66
Conclusion and Discussion 68
CHAPTER 4: TΔIFNAR 70
Hypothesis and rationale 70
Results: Disease Phenotype 71
Thymocyte development is altered in B6.Nba2 lupus-like disease, but is independent of IFN-I T cell stimulation 71
B6.Nba2.TΔIFNAR mice retain classic lupus-like disease presentations of splenomegaly and ANAs 72
Splenic CD4+ T cells subsets and Activated T cells are not altered in TΔIFNAR mice. 73
T cell skewing in TΔIFNAR mice is independent of IFN-I stimulation. 76
Naïve CD4+ T cell cytokine-dependent transition to Th1 and Th2 is not affected 77
by IFN-I 77
Increased TFH cells in B6.Nba2 mice are not a direct result of IFNα stimulation 77
IFN-I directly contributes to limiting the CD8 Tcreg population in lupus like disease 78
Conclusion and Discussion 80
CHAPTER 5: MyΔIFNAR 81
Hypothesis and rationale 81
Results: Disease Phenotype 83
IFN-I signaling in LysM+ myeloid cells affect myelopoiesis in the bone marrow. 83
MyΔIFNAR mice have significantly increased recently activated T cells 85
MyΔIFNAR mice had reduced populations of splenic myeloid cells 87
MyΔIFNAR mice have elevated populations of GC-reaction associated B cells 88
Conclusion and Discussion 90

CHAPTER 6: General discussion and Conclusion 92
Impact of Type I IFN stimulation on B6.Nba2 lupus like disease 92
Working Model Incorporating Experimental Findings 96
Discussion 97
Impact on the field and future directions 101
Potential therputic targets 101
Survival of GC B cells 102
Myeloid derived suppressor cells 105
T regulatory Cells 106
Bibliography 108

List of Tables

Table 1 40
Table 2 45
Table 3 48
Table 4 51

List of Figures

Figure 1 16
Figure 2 23
Figure 3 47
Figure 4 55
Figure 5 56
Figure 6 57
Figure 7 58
Figure 8 59
Figure 9 60
Figure 10 61
Figure 11 62
Figure 12 63
Figure 13 64
Figure 14 65
Figure 15 66
Figure 16 67
Figure 17 68
Figure 18 73
Figure 19 74
Figure 20 76
Figure 21 77
Figure 22 79
Figure 23 80
Figure 24 85
Figure 25 86
Figure 26 88
Figure 27 89
Figure 28 90
Figure 29 97
Figure 30 100

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The Contribution of IFNα-Stimulated Immune Cell Populations to B6.NbA2 Lupus-like Disease

Abstract

By

EMMA JEAN KELLER

Systemic Lupus Erythematosus (SLE) is an autoimmune disease that can present with in a variety of ways, often with chronic inflammation, pain, and fatigue. Unfortunately, the extreme variety of symptoms with which SLE patients present has proven to be a barrier for the successful development of targeted treatments, with only a select few therapies progressing through clinical trials. Interferon α (IFNα) is one of the main cytokines elevated in a majority of SLE patients [1]. IFNα has been also shown to be important for disease development in animal models, however how IFNα is contributing to disease pathogenesis via the direct stimulation of specific immune cells such as B cells, T cells, and myeloid cells remains largely unknown. My research shows that IFNα stimulation of B cells leads to increased B cell activation, increased germinal center B cell survival, increased populations of antibody producing cells, and auto-antibodies. This research also shows that IFNα stimulation of T cells leads to a reduction in CD8+ FoxP3+ Tcregs. Additionally, IFNα stimulation of myeloid cells was found to lead to a decrease in marginal zone macrophages and myeloid-derived suppressor cells that normally provide tolerance. Finally, my research highlights that the action of IFNα is directly tied to the ability of apoptotic pathways to signal correctly. In summary, these findings provide critical insight into the progression of SLE, in addition to identifying new potential therapeutic targets.

CHAPTER 1: Background

What is SLE?

Systemic Lupus Erythematosus (SLE) is an autoimmune disease where the immune system attacks the many different types of cells in the body. In most patients SLE is facilitated by anti-nuclear antibodies (ANAs). Common targets for these antibodies include histone, chromatin, dsDNA, and nuclear proteins, commonly Smith (Sm), Ro, and La. Patients with SLE can present with a variety of symptoms, however a SLE diagnosis typically requires a minimum of four of the up to 11 separate symptoms. Thus, SLE patients as a group are very heterogeneous in their symptom presentation and this heterogeneity has proved to be a barrier to the furthering of SLE care and treatment developments.

Sex-related differences in SLE

Lupus is a predominately female disease and it is 9 times more prevalent in females than in males [2–4]. The average age of disease onset is earlier in females than in males. Disease onset is female often occurs between in the childbearing years, around the ages of 20-30, but disease onset in males tends to be later in life around 45-60 years of age [4–6]. Both sex hormones and sex chromosomes have been shown to contribute to disease progression in separate ways. Sex hormones, estrogen, testosterone, and prolactin among others, have been shown to be dysregulated in lupus. While the exact relationship between hormone dysregulation is still unclear, androgens seem to play an important role. Men with hypogonadism are at a higher risk of developing SLE [7–9], and androgens have been found to be lower in female SLE patients [10–12]. Sex chromosomes also play a role in disease. Toll-like receptor (TLR) 7 is located on the X chromosome, meaning that biologically XX females have two copies. X chromosome inactivation typically shuts

down transcription from one of these two copies, however TLR7 has been shown to escape X inactivation in immune cells [13]. This is significant to lupus because TLR7 is involved in disease development [14–17], and downstream signaling of TLR7 can lead to production of Type I IFNs. In order to control for sex, all studies done in chapters 3-5 were done exclusively using female mice.

Animal models of SLE

NZWxNZB F1 (BW)

The F1 generation cross from a NZW mouse bred with a NZB mouse (BW) is often utilized for its ability to spontaneously develop a lupus-like disease that presents with nephritis [18]. Spontaneous lupus like disease in BW mice, similarly to humans, primarily develops in females [18]. The disease phenotype features splenomegaly, elevated total levels of serum IgG, autoantibodies, mild vasculitis, and nephritis marked by proteinuria and immune complex deposition [18]. Individually, only the NZB mouse presents with spontaneous lupus-like symptoms, however both strains contribute to the symptom presentations of the combined model, with NZW mice contributing increased susceptibility to developing kidney disease in response to anti-glomerular antibodies [19,20]. The NZB mice do not develop fatal kidney disease or proteinuria on their own, but rather show immune complex deposition and complement C3 fixation in the glomeruli [21,22]. They also do produce autoantibodies and increased total levels of IgG and IgM [18]. Lupus-like symptoms in the NZB mouse depend on type I interferon signaling and in the BW mouse model exogenous IFNα can accelerate disease development [23,24].

While the BW mice provide a comprehensive spontaneous disease, there is an obvious disadvantage when utilizing total or selective knockouts, as backcrossing to two separate

backgrounds is often required. Thus researchers have developed New Zealand Mixed (NZM) models to circumvent the need for multiple backgrounds, while still maintaining a majority of the lupus-like disease present in the BW model. Researchers have also created mice who harbor disease-driving loci from either the NZB or NZW strains, resulting in disease presentation on an otherwise non-autoimmune background such as B6. These strains will be discussed in the following sections.

NZM

There are two NZM strains that are common to lupus research, the NZM 2328 and the NZM 2410. The NZM 2328 develop renal disease in two stages consisting of an acute and a chronic stage [25–27]. The NZM 2328 produce anti-dsDNA antibodies and by 9 months of age the strain has a 50% mortality rate [26]. Disease in NZM 2328 mice depends on Type I interferon signaling [28]. The NZM 2410 also produces auto-antibodies and develops glomerular nephritis by way of immune complexes [29,30]. This strain has a shorter lifespan than the NZM2328, reaching a 50% mortality rate already by 6 months of age [31].

B6.Nba2

The B6.Nba2 mouse strain is one of the spontaneous lupus-like disease models that develops symptoms from an autoimmune susceptibility locus on the non-autoimmune B6 background. The autoimmune susceptibility locus comes from distal chromosome 1 of the NZB mouse and is designated *Nba2* for "NZB autoimmunity 2" [19,21]. The B6.Nba2 mice develop autoantibodies against both ssDNA and dsDNA, as well as antibodies against chromatin and histone [19,21,22,32], and as more recently shown, also against RNA binding proteins [33,34]. These mice do not develop proteinuria but

experience immune complex deposition and complement C3 fixation in the glomeruli of the kidneys [35].

B6.Nba2 mice produce an abundance of spontaneous germinal centers (GCs) and develop splenomegaly [35]. Unsurprisingly, the B6.Nba2 mice also present with increased populations of plasma cells (PCs) and memory B cells that develop via the GC reaction. Separate from the GC reaction, B6.Nba2 mice also present with increases in both activated B cells and T cells [35]. Type I interferon receptor (IFNAR)-deficiency ameliorates disease development in B6.Nba2 mice [19].

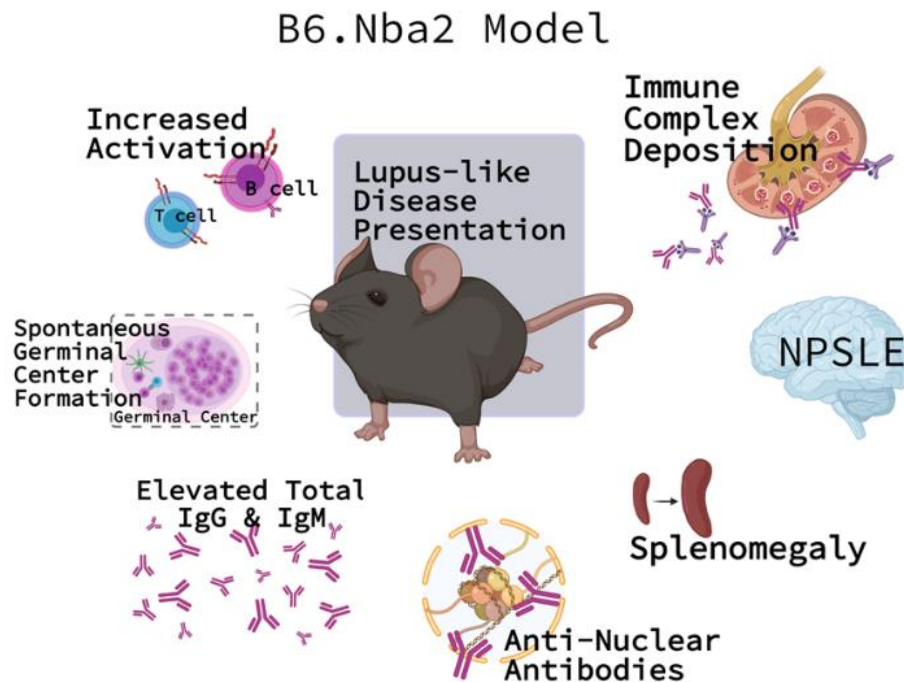

Figure 1: Known presentation of lupus-like disease in B6.Nba2 mice. See text for details.

Recently, it has been discovered that the B6.Nba2 mice also present with a neuropsychiatric SLE phenotype (NPSLE) [34]. This phenotype presents with anxiety and depressive behaviors, as determined by behavioral testing [34]. Browne et al. also

found that anti-dsDNA antibodies positively correlated with reactivity to DWEYS peptide [34], a peptide that mimics binding sites on the glutamate receptor NMDAR (N-methyl-D-aspartate receptor) [36]. The known disease presentation of B6.Nba2 lupus-like disease is shown in Figure 1.

B6.Sle1

The B6.Sle1 model is a more commonly used spontaneous lupus like disease model that develops symptoms due to the expression of an autoimmune susceptibility locus (*Sle1*) on the B6 background [30]. The *Nba2* and *Sle1* loci overlap on distal chromosome 1, but in contrast to the B6.Nba2 mice discussed above, the autoimmune susceptibility locus originates from the NZW mouse and was originally identified using the NZM 2410 mice [30]. Symptoms of the B6.Sle1 mouse model mimic those of the B6.Nba2 model, as they also develop autoantibodies against chromatin and histones (H2A, H2B), and DNA subnucleosomes [37–39]. Lupus-like disease in these mice is accompanied by increased levels of spontaneously active B and T cells and glomerulonephritis and depend on type I interferon signaling similar to B6.Nba2 [37–39].

Murphy Roths Large/ lymphopoliferation (MRL/lpr)

The MRL/lpr mouse model produces a lupus-like phenotype as a result of a mutation in a single gene of the FAS receptor [40,41]. The FAS receptor plays an important role in the apoptosis of both B and T cells and the mutation found in this strain impedes cell death via apoptosis, leading to lymphopoliferation [41]. These mice present with both splenomegaly and lymphadenopathy [42]. In contrast to the spontaneous models described above, the MRL/lpr model of lupus like disease is driven by the FAS mutation that leads to an accumulation of B cells and T cells [43]. Interestingly, while other strains

are protected from disease when deficient for IFNAR, the MLR/lpr mice experience an exaggerated disease state with increased production of autoantibodies and more end organ disease [44].

Pristane Induced Model

Inducible mouse models are also used to study SLE. Pristane is known to induce a lupus-like disease in Balb/c and to a lesser extent in B6 mice [45,46]. The disease is characterized by early monocytosis followed by an appearance of autoantibodies leading to glomerulonephritis and renal damage. Similar to spontaneous models, type–I interferon (IFN-I) is involved in disease progression, as IFNAR-deficient mice fail to develop disease [47]. Interestingly, pristane-induced IFN-I is primarily produced by the monocytes, as opposed to spontaneous production of IFN-I by pDCs in other models [48,49]. The exact subtypes of IFN-I produced in response to pristane have not yet been defined, however it has been shown that pristane induced IFN-I production is TLR7/MyD88 dependent [50].

BXSB

The BXSB model of lupus like disease is unique in that it involves translocation of a portion of the X chromosome onto the Y chromosome, where it does not naturally exist. This Y-derived locus chromosome is referred to as *"Yaa"* and it aids in the development of lupus-like disease in this model [43,51]. After the BXBS model was created, it was found that Yaa contains a second copy of TLR7 which drives disease in this model[15]. This is notable for a few reasons. First, TLR7 is located on the X chromosomes, meaning that biological females (XX) have a second copy compared to biological males (XY), and SLE is a disease that affects females significantly more frequently than males. Secondly,

one of the main outcomes of TLR7 signaling is the production of IFN-I and upregulation of interferon stimulated genes (ISGs) [52].

Involved cell subtypes

Plasmacytoid dendritic cells (pDCs)

Plasmacytoid dendritic cells (pDCs) are known to be involved in SLE, primarily due to the cytokines they produce. The pDCs are the main producers of IFN-I, which is produced in response to toll-like receptor (e.g. TLR3, TLR7, TLR8, TLR9) cross-linking [52–54]. Both overexpression of TLR7 and mutations in TLR7 are found in SLE populations[15,55–57]. IFN-I has been found to be important to SLE disease, and its particular impact is discussed later in this chapter [1]. Notably the pDCs have a capacity to produce large quantities of all subtypes of IFNα [54]. In addition, pDCs produce both IL-6 and TNF-α, with potential immune modifying effects as a result [58–60]. For example, autocrine TNF-α stimulation can speed pDC maturation [59], while production of IL-6, in combination with IFNα, can mediate development of B cells leading to increased populations of plasma cells and elevated Ig secretion [60].

In SLE in particular, the IFN-I production capability has made pDCs the target of ablation and knockout models. B6.Nba2 mice that expressed the diphtheria toxin receptor (DTR) under the control of a pDC-specific promoter (BDCA2) selectively depleted pDCs upon receiving diphtheria toxin [61]. After 6-8 weeks of pDC depletion these mice showed significant reduction in lupus like disease including reduced presence of IFN-I induced gene transcripts signifying reduced levels of circulating IFNα, decreased anti-chromatin autoantibodies, and decreased levels of activated B and T cells, GC B cells, TFH cells, and splenic PCs [61]. The DT-dependent depletion of pDCs leading to decreased disease has also been shown in the BXBS.DTR mice, that similarly benefitted

by reductions in autoantibody production, activation of immune cells, severity of glomerular nephritis, and decreased ISG expression [62].

Moreover, albeit limited in quantity, patient data in regards to pDC involvement in SLE also supports a pathogenic role for pDCs in disease. Clinical studies have shown that a majority of IFNα producing cells in SLE express BDCA2, a pDC surface marker, and separately that pDCs accumulate in skin lesions in SLE and cutaneous lupus erythematosus (CLE) patients [63,64]. Further studies have shown that pDCs will activate in response to self-DNA containing peptide complexes produced by neutrophils and anti-dsDNA complexes [65,66]. This data supports both a method for pDC activation in SLE in response to self-antigen, and a major role for pDCs as IFNα producers in driving disease.

B cells

B cells are another immune cell type that plays a role in lupus disease development. B cells are often thought of as the origin of antibody manufacturing and production during an immune response. B cells can develop into plasma cells (PCs) or memory B cells, with the ability to produce antibodies crucial for targeting pathogens for elimination [67]. In fact, PCs have been known for their ability to secrete antibodies for almost 70 years [68]. While in the context of disease, antibody production is often considered a positive step towards resolution, this is not true in SLE as PCs also produce autoantibodies.

In SLE, there is a high prevalence of anti-nuclear antibodies (ANAs) that bind to the nuclear content of one’s own cells [69]. While cases of ANA negative lupus do exist, they are rare, with over 90% of SLE patients being positive for ANA [69–71]. Common targets for ANA found in SLE patients include; dsDNA, chromatin, histone, Sm (nuclear

protein), Ro (nuclear protein), La (nuclear protein), and RNP [72–79]. Patients can also develop autoantibodies targeted against phospholipids, that can lead to the development of anti-phospholipid syndrome as well as thrombotic events [80,81].

Plasma cells responsible for antibody production, can initially develop via the extrafollicular (EF) response, but after the initial response they typically develop as a result of the GC reaction [68,82–84]. It is therefore unsurprising that many spontaneous lupus mouse models have a significant increase in spontaneous GC formation, along with increased levels of PCs [19,21,25,30,39,42,85,86]. Children with SLE have been found to have increased circulating levels of cells expressing a pre-GC B cell phenotype and cells expressing a pre-PC phenotype, indicating that B cells contribute significantly to human disease in the same processes as seen in mice [87].

Plasma cells in particular are necessary to drive disease, which is confirmed by disease amelioration in both the BW and MRL/lpr models after administration of bortezomib, which targets and depletes PCs [88]. In humans, a monoclonal antibody therapy that targets CD20, Rituximab, can deplete CD20+ B cells but notably does not deplete long-lived plasma blasts [89,90]. This therapy has been successful in treating RA, an autoimmune disease that shares some overlap with SLE [91], but has had a varied amount of success in treating SLE [92,93]. Recently, a meta-data analysis did find that Rituximab treatment was able to significantly reduce the use of prednisone in SLE patients and reduce proteinuria levels in lupus nephritis patients [94].

B cells can also act as antibody presenting cells (APCs). Similar to other APCs (such as dendritic cells and macrophages), B cells constitutively express MHC II, which enables both resting and active B cells to present antigen to CD4+ helper T cells [95–97]. Antigen

presentation via MHC II is known to play a an integral role in SLE, as spontaneous lupus mice, both B6.Nba2 and B6.Sle1, bearing a MHC II knockout fail to develop disease [98].

T cells

Literature has shown that the T cell compartment plays an important role in lupus, both in humans and murine models. Precursor T cells enter the thymus as CD4- CD8- double negative (DN) cells, transition to a CD4+ CD8+ double positive stage, and later are released as naïve cells expressing either CD4 or CD8, if they can first make it through the gauntlet designed to remove autoreactive and non-functional cells [99–102]. The cells then circulate and home to secondary lymphoid organs where they typically reside until activated by antigen-presenting cells expressing peptide antigens in the context of MHC class I (CD8) or MHC class II (CD4). Traditionally, CD8+ T cells have the ability to kill other cells in a cytotoxic manner, while CD4+ T cells provide help via cytokine release and play a critical role in providing differentiation signals to B cells and setting them on the path to become antigen producing cells [103,104]. Today we know that the family of T cells consists of several subsets of both CD8+ and CD4+ T cells, largely categorized by the cytokines they secrete.

CD4+ T cells

CD4+ T cells, also known as T helper (Th) cells, can be divided into several subsets depending on their cytokine production profile and functionality. Naïve CD4+ T cells can further develop into Th1, Th2, Th9, Th17, Th22, TFH, or Tregs [105–112]. Naïve CD4+ T cells develop into these subsets primarily as a result of the local cytokine milieu [108,113–116]. The differentiated Th cells then contribute to the cytokine milieu by secreting several different cytokines, specific to the differentiated cell subset, shown in Figure 2. Th1 cells are known for their production of IFN-γ, and Th2 cells are known for their production of IL-4 and IL-13 [117]. Logically after several subsets were discovered, the next subsets were named based on the cytokines they produce. Th9, Th17, and Th22 produce IL-9, IL-17 and IL-22 respectively [118,119]. Some of the Th subsets are better known, and named, for the specific role they play. T Follicular Helper (TFH) cells are important to providing help to GC B cells in the follicles during the GC reaction. T

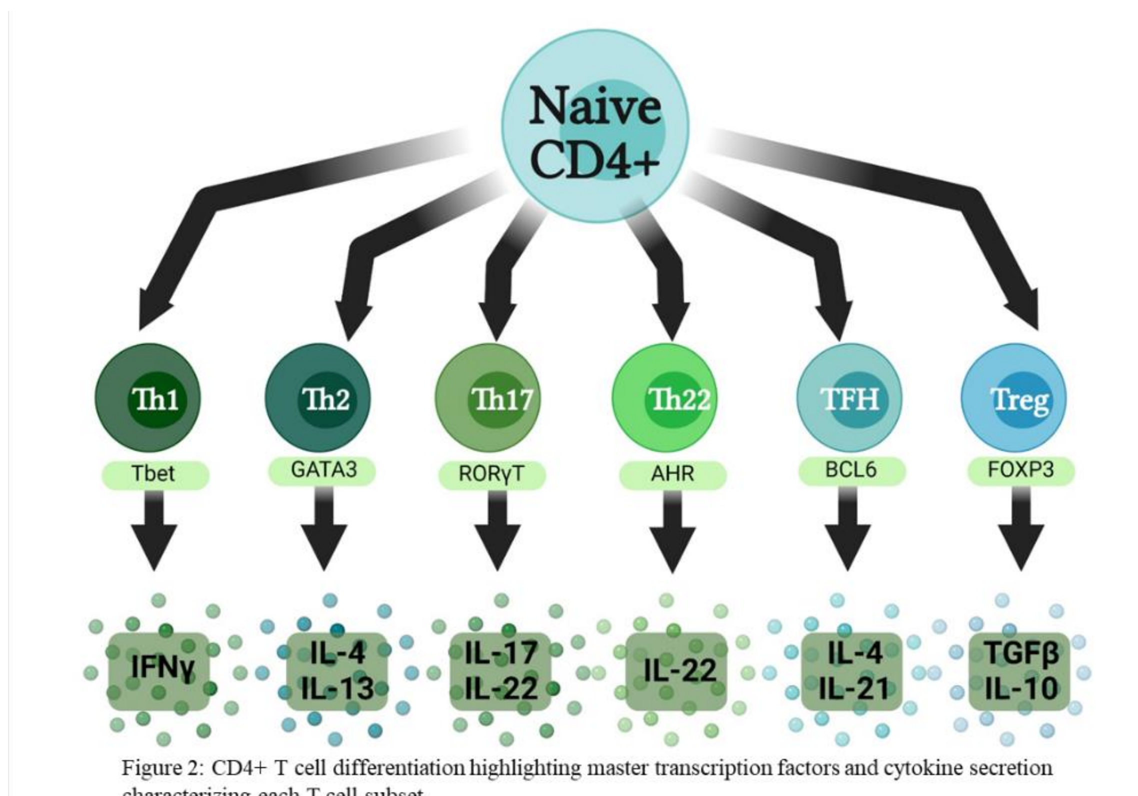

Figure 2: CD4+ T cell differentiation highlighting master transcription factors and cytokine secretion characterizing each T cell subset.

regulatory (Tregs) cells function predominately as gate-keepers, as their primary function is to downregulate inappropriate immune responses, to both self and non-self-antigens, and maintaining tolerance

Most T helper subtypes upregulate a subtype-specific master transcription factor. The known master transcription factors are; TBET for Th1, GATA3 for Th2, RORγT for Th17, AHR in Th22, BCL6 for TFH, and FOXP3 for Tregs [107–112] . Identifying the expression pattern of master transcription factors is an accurate, and often preferred, way to identify particular subtypes of T cell, as cytokine production may be low and difficult to measure without significant manipulation of the cells ex vivo.

CD8+ T cells

CD8+ T cells are also known as cytotoxic T cells, and recent studies suggest that CD8+ T cells can differentiate into a variety of cytotoxic effector cells, secreting different cytokines. Similar to CD4+ T cells, cytotoxic T cells can be categorized via the cytokines they produce and follow similar naming conventions to those of CD4+ Th cells. Type I CD8 T cells (Tc1) are known for their production of IFN-γ, whereas Tc2 cells are known for producing IL-4 and IL-5 [120]. Both subsets maintain their cytotoxic abilities, however there is evidence that their ability to kill is impacted by target cell type and cytokine treatment of the target cell type [121]. In a study using keratinocytes as a target cell, researchers found that only cytotoxic CD8+ cell were able to kill the keratinocytes, and when the keratinocytes were treated with IFNγ, they could only be killed by the Tc1 cells, while the Tc2 cells were unable to kill [121].

Dysregulation in lupus patients and animal models of lupus

CD4+ Effector T cells, particularly in patients with high disease activity, and memory T cells are expanded in SLE patients [122,123]. Intuitively, the T cell population primarily

suspected of dysregulation in SLE is the T follicular helper (TFH) cell population. This is due to the co-stimulation TFH cells provide to GC B cells in the germinal center reaction, which leads to the creation of antibody-producing plasma cells. As a positive ANA is the most common presentation in SLE, it is reasonable to expect TFH cells to be dysregulated. Literature shows that indeed, SLE patients present with increased populations of circulating TFH cells, capable of producing IL-21 and positively associated with disease activity [124–126]. Increased populations of circulating T cell subsets cells have further been shown to contribute, in a CD40L dependent manner, to increased ANAs [127].

Interestingly, SLE patient studies show decreased populations of TH1 and Tregs, with an increase in TH17 cells [128,129]. The role of Tregs in the immune system is centered on maintaining tolerance, so it is unsurprising to see Tregs decreased in an autoimmune disease such as SLE. Patients with active SLE have been found to have decreased levels of circulating Tregs, and in pediatric patients, a decrease in Tregs was found to be correlated with an increase in ANAs [130,131]. A connection between autoreactive B cells and Tregs is supported by a study in which patients were treated with Rituximab, and subsequently found to have increased expression of *FOXP3*, the master transcription factor for Tregs [132]. This same study found that patients who remained in remission maintained increased *FOXP3* mRNA [132].

SLE patients have also been documented to have increased ratios of Th17/Th1 and Th17/Tregs when compared to both healthy controls and patients with anti-phospholipid syndrome [133]. Peculiarly, IL-17 production was found in a population of CD4- CD8- double negative (DN) T cells in the peripheral blood of SLE patients [134]. This

population of peripheral blood DN T cells had already been found to be significantly expanded in SLE patients prior to the discovery that the DN T cells were a source of IL-17 [134,135]. Consistent with the increased populations of Th17 cells, patients with SLE are also reported to have increased levels of serum IL-17, the main cytokine produced by Th17 cells [128,129,136]. IL-17 itself is associated with the development of nephritis among lupus patients and IL-17-producing DN T cells were found in kidney sections from patients with nephritis, along with other IL-17+ T cells [134]. In the MRL/lpr model, researchers were able to reduce kidney damage by reducing the Th17 population using an adenovector that increased IL-2, and hence reduced the Th17/Treg ratio [137]. Not only have IL-17 producing cells been linked with nephritis, but IL-17 has been associated with a loss of B cell tolerance in another autoimmune disease, myasthenia gravis [138]. This indicates that Th17 cells could contribute more broadly to disease pathogenesis in addition to their role in nephritis.

To further understand the function of different T cell subsets, a transcriptome analysis was performed on purified SLE patient CD4+ and CD8+ T cells. Transcript distribution showed increases in CD4+ transcripts found in stimulated cells and a decreased population of exhausted CD8+ T cells [139]. The study showed that opposite to cases of viral infections, larger populations of exhausted CD8+ T cells were associated with better clinical outcomes in SLE [139]. Further supporting a role for CD8+ T cell exhaustion in SLE, studies of SLE patients with lupus nephritis (LN) showed that kidney infiltrating CD8+ T cells expressed decreased levels of exhaustion markers and that these less exhausted CD8+ T cells were associated with an increase in the disease-associated chemokine gradient in LN patients [140,141].

Similarly, mouse models of lupus present with abnormal T cell compartments. Murine models show an increase in activated T cells [33,39,61,85], increased Effector /Memory (Eff/Mem) T cells, and decreased CD8+ T cells [33,61,85]. In the B6.Sle1b mice, production of both IFNγ and IL-4 by T cells was increased [39,142]. Other murine models, including the BW and MRL/lpr models were shown to express decreased IL-2 production, associated with the increased IFNγ [143–145].

As referenced above TFH cells, play a critical role in the GC reaction. In order to undergo activation and proliferation in the GC reaction, B cells carrying bound antigen need to receive stimulation via a successful CD40/CD40L interaction with a TFH cell [146–150]. Using chemokine gradients, TFH cells, identified as CXCR5+ and PD1+ double positive, can migrate to the B cell follicle to interact with B cells [151,152]. The expression of *BCL6/Bcl6* in TFH cells is sufficient to program the TFH cells for appropriate migration [108,153]. Once interacting with B cells at the TB border, TFH cells will provide costimulation signals and cytokine help to those B cells presenting a peptide via MHC II that make a strong, productive interaction with the TCR [154]. These costimulatory signals include CD40/CD40L and ICOS/ICOSL, and the main cytokines produced by TFH cells include IL-21 and IL-4 [155–158]. Blocking CD40/CD40L or ICOS/ICOSL interactions [159,160] or removing MHC II expression has been shown to reduce disease development in several lupus mouse models, further supporting a role for TFH cells in disease pathogenesis [98,161–163].

Myeloid cells

The immune cells previously discussed are not the only immune cells involved in SLE. Myeloid cells also play a role in lupus. There are a variety of different myeloid subsets.

These subsets develop in the bone marrow, beginning as hematopoietic stem cells (HSCs), developing into multipotent progenitor (MPP) cells, and then to common myeloid progenitors (CMP), which can then differentiate into different subsets of myeloid cells [164]. This includes neutrophils, monocytes, and macrophages which have been studied more extensively in lupus, as well as eosinophils and basophils, which will not be discussed in detail here. Along with the discovery of an increased ISG signature in SLE patients, research by Virginia Pascual's lab concomitantly showed an upregulation of a granulopoiesis-associated gene signature in pediatric SLE patients including genes such as MPO, elastase, and MMPs [1], suggesting a prominent role for granulocytes including neutrophils and monocytes, which will be further discussed below.

Neutrophils

Researchers have hypothesized that neutrophil extracellular traps (NETS), extruded during neutrophil death, are the source of nuclear content utilized to make ANAs in disease. NETs contain a mesh-like combination of elastase, chromatin, histones, dsDNA, and granulocytic proteins [165–168]. Considering the overlap between antigens found in NETs and the targets of auto-antibodies found in SLE patients, NETs present a reasonable source of self-antigen.

Neutrophil involvement in SLE is not limited to a possible source of self-antigen. Neutrophils have also been shown to contribute to vasculitis via NET formation in mouse models of SLE, leading to inflammation and tissue damage that can be reduced when mice are treated with peptidylarginine deaminase that reduces NET formation [169–171]. The contribution of NETs to vasculitis is further confirmed by vasculitis in patients presenting with elevated levels of antibodies specific to self-antigen found in NETs

[172,173]. Vasculitis can also contribute to inflammation resulting in damage of the kidneys [169–173].

Monocytes

The pristane-induction model used in BALB/c mice, discussed in a prior section, is a model of lupus-like disease that is known to develop from increased levels of IFN-I produced by the Ly6C+ monocytes, rather than the pDCs [45,47]. This model presents with a particular accumulation of monocytes, due to an increase in these monocytes leaving the bone marrow and accumulating in the circulation and periphery [174]. In the pristane model, the accumulation of Ly6C+ monocytes is associated with increases in auto-antibodies and IFNα levels [174]. While the pristane model is well known for the accumulation of monocytes, monocytes have been shown to play significant roles in other mouse models of lupus-like disease where they are not the main IFN-I producers[175–178].

Macrophages

Similar to other immune cells, macrophages also can present with distinct subtypes and effector functions. Two common subtypes are referred to in the literature as M1 and M2, with M1 described as inflammatory and M2 as resident and involved in maintenance [179]. Macrophages have been reported to have the ability to switch between the different subtypes when necessary [180]. SLE patients have been found to have increased differentiation of monocytes to macrophages, compared to healthy controls [181]. Both mouse and patient studies report a skewing of macrophages in SLE towards increased M1s and decreased M2s, and a positive correlation was found between levels of M1 cells and disease severity in SLE patient [177,182].

An additional source of self-antigen in SLE is hypothesized to be apoptotic cells in the splenic follicles. Apoptotic cells in the follicles are typically removed by marginal zone (MZ) macrophages. Studies done in the B6.Sle1/2/3, a triple congenic strain including not just *Sle1* described above, but also two separate loci names *Sle2* and *Sle3*, and the BXD2, which presents with autoimmunity, models showed that the clearance of apoptotic cells by MZ macrophages was significantly reduced as a result of elevated levels of IFN-I [183]. The prevention of MZ macrophage apoptotic clearance is mediated by a dense accumulation of IFN-I producing pDCs in the splenic perifollicular region where MARCO+ MZ macrophages, able to clear apoptotic cells, would normally be [183]. This displacement is in part mediated by IFN-I, which disrupts cell surface interactions that normally lead the MZ macrophages into the perifollicular region to clear apoptotic cells [183].

Myeloid Derived Suppressor Cells (MDSCs)

Previously published data shows that female BW mice have constitutively decreased populations of immunosuppressive Gr1 high, Ly6G+, CD11b+ cells [175]. These cells, when studied in BW males, are able to target B cells and reduce ANA production [175]. These cells have been called “Myeloid Derived Suppressor Cells” (MDSCs). The expansion of the MDSC population has been shown to be a direct effect of testosterone, as castrated males supplemented with 5α-dihydrotestosterone were able to reconstitute the MDSC population, while those receiving no supplemental hormones could not [175].

Cytokines involved in SLE pathogenesis

As SLE is an autoimmune disease it is not surprising that dysregulation of cytokines often accompanies disease presentation. Several cytokines have been found to plan an important role in disease progression.

IFNα

Type I interferons (IFN-I) have been found to play a central role in SLE. In the early 1980s a patient study reported an increase of IFN-I in the serum of SLE patients and that this increased serum IFN-I was positively correlated with both disease activity and dsDNA autoantibodies [184]. Type I IFNs includes α, β, ε, ω, δ, τ, ζ and κ, all of which signal through IFNAR [185,186]. Very little is known about the effect of IFN ε, ω, δ, τ, ζ and κ. However of all the type I IFNs, IFNα has been shown to have the greatest contribution to lupus-like disease at this time [187–191].

By the early 1990s, clinicians had reported cases where patients receiving exogenous IFNα treatments developed elevated levels of ANAs and in some cases a SLE-like syndrome [192–195]. IFNα therapy is still used to treat both Hepatitis C Virus (HCV) and hematologic malignancies, and has been shown to initiate an autoimmune response in patients receiving this treatment at a rate of 4-19%, further confirming the earlier case studies showing that IFNα alone could initiate an autoimmune response [192]. While patients who experience an autoimmune response due to IFNα treatment do not all experience SLE-like symptoms, this response indicates that IFN-I plays an important role in SLE [196].

Further data published in 2003 shows that PBMCs from SLE patients showed an upregulation of interferon stimulated genes (ISGs) [1]. This increased ISG signature was

unique to SLE patients when compared to both healthy controls and rheumatoid arthritis (RA) patients [1]. The link between IFN-I and SLE was found by other groups, and shortly thereafter it was discovered that IFN-I was significantly correlated with active SLE, and particularly with ANA production [197–200]. IFN-I has since been found to correlate with Sjögren's Syndrome, an autoimmune disease driven by auto-antibodies in which common symptoms can overlap with SLE presentations [201,202].

As discussed previously, IFNα is predominantly produced by the pDCs in SLE. When IFNAR is stimulated it begins the signaling cascade through phosphorylation of STAT1 and STAT2 by Jak1 and Tyk2 [203]. This results in the activation of other downstream factors, nuclear translocation, and the activation of interferon sensitive gene factors (ISGFs) and interferon response factors (IRFs) [203]. These ISGFs can then lead to the activation of interferon-stimulated response elements (ISREs) and the transcription of ISGs [203]. Of the Type I IFNs, IFNα has been shown to have the greatest contribution to lupus-like disease at this time [187–191]. Interestingly, pDCs can respond to IFNα levels in an autocrine manner [204–208].

Genetic mutations found in the population also support a role for IFN-I in SLE. Polymorphisms in the genes for *IRF5* [209,210] that leads to increased IFN-I production and polymorphisms in *STAT4* [211] , *TYK2* [209], and *IRAK1* [212] that lead to a greater sensitivity to IFN-I have been found to be risk factors for SLE.

When IFN-I was studied in mouse models of SLE, it was found by a variety of researchers that IFN-I was driving lupus-like disease. Total IFNAR knockout (KO) mice on the B6.Nba2 [19], (B6.Nba2 x NZW) F1 [19], NZM2328 [28], and NZB [23]

backgrounds were shown to ameliorate spontaneous lupus-like disease. IFNAR KO mice were also protected from lupus-like disease induced by administration of pristane [49]. This indicates that not only are spontaneous models of lupus-like disease driven by IFN-I, but induced models required functional IFN-I signalling to succesfully induce disease. The notable exception is IFNAR KO MRL/lpr mice that experience more severe disease [44].

IFNγ

Unlike IFN-I, IFNγ is a Type II IFN, which signals via the IFNγ receptor (IFNGR) comprised of two subunits: IFNGR1 and IFNGR2 [213,214]. Similarly to IFNAR signaling, JAK1 is involved in the downstream signaling of IFNGR, however JAK2, and not Tyk2, is activated downstream of IFNGR activation [215,216]. IFNγ signaling results in the formation of a STAT1 homodimer, rather than a STAT1/2 heterodimer as seen in IFN-I signaling [217]. Thus, while some overlap exists between the subunits in the signaling pathways for Type I and Type II IFNs, they are still distinctly different.

IFNγ, like IFN-I, has also been shown to have a role in SLE. Lupus prone mice have been shown to have significantly elevated levels of IFNγ, particularly later in disease [218–222]. These elevated levels of IFNγ are not only found in serum and lymph nodes but also in the kidneys of lupus prone mice [219]. Recently, IFNγ has been shown to specifically contribute to renal damage and immune complex deposition, as lupus-prone mice lacking the IFNGR were significantly protected from the kidney damage that typically develops in the B6.Sle1 model of spontaneous lupus like disease [223]. Notably, IFNγ is secreted by TH1 cells more so than any other T cell subset, and has been linked with the T cells that infiltrate the kidney [221].

IL-6

Another cytokine produced by the pDCs, IL-6, has also been shown to play an important role in SLE. Genetic studies have revealed that SLE patients express significantly different allelic patterns in the region of the Il6 gene, resulting in an increase in the stability of the *Il6* mRNA transcripts and subsequently increased IL-6 secretion [224]. This observation was supported by another study in which serum of SLE patients was further shown to contain elevated levels of IL-6, with higher levels being more strongly associated with active disease, as compared to inactive disease [225]. Mouse models of lupus further confirm a role for IL-6. Both the MRL/lpr and the B6.Sle1.Yaa mice (B6.Sle1 mice expressing the *Yaa* locus from BXSB mice) have been reported to have increased levels of IL-6 as they age and experience increased disease [226–230].

IL-6 plays a crucial role in the differentiation of TFH cells that then provide the necessary costimulatory signals to initiate B cell activation in the GC reaction, as well as immunoglobulin production and secretion [231,232]. It is therefore unsurprising that in SLE, where ANA production is a primary presentation of disease, IL-6 plays a role in disease. The effect of IL-6 is directly on B cells, as *ex vivo* splenic B cells cultured with IL-6 upregulated antibody production compared to splenic B cells cultured without it [233]. Finally, research suggests that also macrophages can produce IL-6 contributing to the increase in antibody production in the BW model, as both macrophage depletion and treatment with IL-6 neutralizing antibodies were able to decrease antibody production similarly [234].

IL-10

IL-10 is often considered to be an “anti-inflammatory” cytokine but in reality IL-10 can act in both pro- and anti-inflammatory ways. Particularly relevant in the context of lupus,

IL-10 can help initiate PC transition of B cells that have been CD40 activated, as well as aid in growth and differentiation in B cells [235,236]. Unsurprisingly, SLE patients present with elevated levels of serum IL-10, with elevated serum IL-10 correlated with increased disease activity [237–239]. Conversely, B6.Sle1/2/3 and NZM models of spontaneous disease showed that an increased in IL-10 was able to help prevent disease progression by delaying the production of ANAs and the onset of nephritis [240]. Studies done in the BW model of lupus show that IL-10 can actually act in different ways to both increase and decrease disease [241,242]. While it is clear that IL-10 plays a role in lupus, research must consider that the contribution of IL-10 may differ based on time, place, and the other contents of the cytokine milieu.

Current treatment options for SLE

Due in a large part to the wide span of heterogeneous disease presentation, SLE treatment options remain more limited than many other diseases. In fact, only one new therapeutic, Belimumab, has been approved specifically for SLE in the past 60 years [243]. Therefore it has been necessary to continue to utilize a range of treatments, most of which were not developed with SLE as the sole target. The treatments commonly utilized to control SLE, as well as SLE specific treatment, and a selection of SLE specific treatments currently in clinical trials are discussed below.

General immunosuppression

As SLE is a disease in which the patient experiences chronic inflammation, general immunosuppression has been highly utilized to help control disease. Gluccocorticosteroids, in particular prednisone, are commonly utilized in SLE for general immunosuppression [244]. While many SLE patients will be prescribed prednisone to control disease, this medication regime has drawbacks. Prednisone can help reduce

disease activity in SLE patients and promote apoptosis in cell subsets, including the pDCs, however it cannot protect them against organ damage or induce remission [245].

Anti-malarials/hydroxychloroquine

Another commonly used treatment in SLE comes from medications initially developed as anti-malarials; chloroquine (CQ) and hydroxychloroquine (HCQ) [246–248]. Hydroxychloroquine and chloroquine are generally considered safe, although in rare cases they can lead to retinopathy [249,250]. CQ and HCQ work by preventing the fusion of the phagosome and the lysosome to create the phagolysosome wherein target structures are degraded in preparation for presentation [251–253]. In the context of an autoimmune disease such as SLE this is important to prevent the ultimate presentation of self to the immune system and the creation of autoantibodies.

Another way anti-malarials may be working to prevent disease is through interrupting the signaling ability of endosomal TLRs, such as TLR7 and TLR9. Reviewed in depth by Schrezenmeier and Dörner, there are several mechanistic possibilities that would enable anti-malarials to interfere with endosomal TLR signaling [254]. One possible way is that CQ and HCQ are altering the endosomal pH, creating an environment wherein TLRs cannot signal due to processing inabilities [255]. A second possibility is that CQ and HCQ are directly binding to nucleic acids and disrupting the TLR ligand interactions, resulting in decreased signaling [256,257]. Also discussed in the review is the possibility that the mechanism of action of CQ and HCQ is by inhibiting the STING pathway that provide a major contribution to IFN-related responses [258].

Anti-BAFF antibodies

The first biologic treatment for SLE came in the form of Belimumab, a monoclonal antibody (mAB) directed against B cell activating factor (BAFF), also known as B cell lymphocyte stimulator (BLyS) [243,259]. Belimumab, by binding BAFF/BLyS, is able to reduce the survival of B cells and decrease antibody production [260].

Anti-IFNAR antibodies

Monoclonal ab therapy targeting the Type I IFN Receptor (IFNAR), anifrolumab, has been in clinical trials to prove efficacy. Anifrolumab leads to internalization of IFNAR and therefore inhibition of STAT1/STAT2 activation and reduced induction of ISGs, including IFNα's, effectively breaking the IFN-I amplification cycle [261]. Results of the most recent phase III studies (TULIP-1 and TULIP-2) show that anifrolumab is able to reduce disease activity and allow for the reduction of oral steroid dose better than placebo [262]. These studies also showed that anifrolumab is safe, with a rate of adverse events similar to placebo treatment [262].

Anti-CD20 antibodies

While not specifically FDA approved for SLE, the anti-CD20 mAB rituximab, has been utilized off label to help treat SLE. Rituximab depletes B cells, which as discussed above, play a major role in SLE disease development [263]. Surprisingly, rituximab failed to meet primary endpoints in clinical trials for SLE, despite succeeding in significantly reducing detectable anti-dsDNA antibodies and increasing complement C3 compared to placebo [263]. A more recent meta-analysis of rituximab treatment was more promising, finding that treatment significantly decreased the necessary prednisone dose in patients and significantly decreased proteinuria in lupus nephritis patients [94]. While rituximab is not specifically approved for SLE, it is FDA approved to treat RA. As arthritis symptoms

are relatively common in SLE, rituximab will likely remain an off-label treatment option, particularly for patients with arthritic symptoms.

Study Plan

Rationale

In order to advance research, treatments, and standards of care for SLE, the field is in need of a scientific way to further separate patient populations. Many researchers in the field believe that SLE is likely not one specific disease, but rather a collection of similar diseases that may present similarly but are in fact driven by different mechanisms. This presents significant hurdles to discover effective therapeutics. Many therapeutic trials find that they are successful in treating a subset of patients, but not all. *It would be ideal and most likely increase the number of effective drugs if researchers could define specific patient sub-populations to target in specific drug trials.*

IFN-I has been shown to be associated with SLE and to correlate with disease activity and ANA production [1,197–200]. Moreover, all of pathogenic cell subsets described above are known to respond to IFN-I in different ways. Still, ***despite knowing that IFN-I is sufficient to drive lupus disease, the IFN-I-dependent pathogenic contribution(s) to SLE disease by different immune cells remains unknown.*** My study aim was therefore to understand the relative contribution to IFN-I-driven lupus-like disease of three specific cell types.

Hypothesis

IFN-I stimulation of B cells, T cell, and myeloid cell subsets is contributing to development of disease pathogenesis and the contribution of each of the different cell subsets leads to distinct symptom presentations.

Cell specific IFNAR knockout mice

To determine the pathogenic disease contribution of different immune cells in response to increased IFN-I, we utilized cell-specific IFNAR knockouts for the cell types in which we wanted to establish IFN-I stimulated contributions. We obtained $IFNAR^{flx/flx}$ mice in which the second exon was genetically modified to contain two flanking loxP sites; targets for cre recombinase-mediated excision[264]. The cre recombinase was expressed under the control of cell type specific promoters normally controlling the expression of *Mb1, Cd4* and *LysM*, allowing us to target B cells, T cells, and myeloid cells (including monocytes, macrophages, and neutrophils), respectively [265–267]. When the selected promoter is activated during cell development, the cre recombinase is produced and the LoxP-flanked region is excised resulting in premature translation stop and a nonfunctional protein. All genetically modified models used were transferred to the B6.Nba2 background before establishment of specific conditional KO lines. B cell specific IFNAR knockouts were designated as BΔIFNAR mice, T cell specific IFNAR knockouts as TΔIFNAR mice, and myeloid cell specific knockouts as MyΔIFNAR mice. It should be noted that as T cells undergo a CD4+ CD8+ double positive stage during thymic development, the cre recombinase is activated, subsequently affecting both CD4 and CD8 single positive cells [267].

CHAPTER 2: Methods

Study Animals

All animals were housed in specific pathogen free housing at the Lerner Research Institute at the Cleveland Clinic. All Mb1-cre, CD4-cre and LysM-cre driver strains and IFNAR flx/flx mice were backcrossed from a C57Bl/6 (B6) background onto the B6.Nba2.ABC background upon arrival [85]. Offspring of the first cross were screened

for D1Mit36, D1Mit47, D1Mit113, and D1Mit209 markers, as these have previously been shown to present differently in NZB and B6. Progeny expressing the NZB-genotype at all four D1MIT markers were backcrossed to the B6.Nba2 background to generate mice homozygous for the Nba2 locus.

At two weeks of age mice were ear clipped and genotyped using the primers in Table 1. Female study mice and control littermates were set aside for study and harvested at four or nine months of age. Female mice were used for all studies as the female mice develop a more severe phenotype [32,34].

Target Name	Primer Sequence 5'-3'	Primer Sequence 5'-3'	Primer Sequence 5'-3'
IFNAR Flx	TGC TTT GAGGAG CGT CTG GA	CAT GCA CTA CCA CACCAG GCT TC	TAG CCC CAG GGT AGT TAACTC TTG A
MB Cre	CCC TGT GGA TGC CAC CTC	GTC CTG GCA TCT GTC AGA G	
LysM Cre	CCC AGA AAT GCC AGA TTA CG	CTT GGG CTG CCA GAA TTT CTC	TTA CAG TCG GCC AGG CTG AC
CD4 Cre	GTT CTT TGT ATA TAT TGA ATG TTA GCC	CTT TGC AGA GGG CTA ACA GC	

Table 1: Sequences of genotyping PCR primers. Primers were ordered from Integrated DNA Technology and suspended in RNAse free water for use at a concentration of 10uM.

Harvest and Processing of Tissues

Older mice were bled monthly for serum starting at two months of age. All study mice were bled prior to harvesting, and serum and PBMCs were obtained from those samples. Mice were sacrificed by cervical dislocation and spleens were obtained from all animals. For BΔIFNAR and MyΔIFNAR studies bone marrow was also harvested, and for TΔIFNAR mice, the thymi were harvested.

Spleens were weighed following removal, and a center section was removed and preserved in OCT. Another portion of the spleen was mashed in 300uL of 1xPBS and then incubated for 10 minutes at 37˚C. Samples were centrifuged and the spleen

supernatant, containing released cytokines, was removed and stored for further experiments. Red blood cells were removed from the sample using the ACK protocol (described below) and split into two samples. One portion was frozen at -80˚C for RNA isolation. The other was suspended in western blot lysis buffer and frozen at -20˚C for future protein analysis. The remaining spleen was made into single cells, red blood cells were removed, cells suspended in 1x PBS, counted using the Beckman Coulter Cell Counter, and plated for flow cytometry using the protocol detailed in a section below.

Bone marrow was acquired by flushing the left femur and tibia with 1x PBS. Red blood cells were removed and cells were stained for use in flow cytometry. The left kidney was split into two coronal sections. One section was flash frozen and preserved in OCT and the other was fixed in formalin and later used for paraffin embedding. The thymus was removed, made into single cells, and red blood cells were lysed. Thymocytes were used for flow cytometry and an aliquot was frozen and stored at -80˚C for RNA.

Red Blood Cell (RBCs) Removal

In order to remove RBCs from single cell preparations, the cell pellet was re-suspended in ammonium-chloride-potassium (ACK) lysis buffer (0.15M NH_4Cl, 0.01 M $KHCO_3$, 0.2 mM EDTA; pH 7.3± 0.1) as previously described [33]. Cells were incubated at (room temperature) RT for 5 minutes and then quenched with an equal volume of 1xPBS. Cells were centrifuged and supernatant removed via aspirator.

Immunohistochemistry

Half of a kidney per mouse was preserved in 10% formalin and then transferred to 80% ethanol prior to embedding. Kidneys were embedded in paraffin, sectioned (5 µm), and stained with hematoxylin and eosin (H&E) by the Histology Core at the Cleveland Clinic Lerner Research Institute. Unstained slides were used for PAS and CD4/CD8 staining, as

described below. The other half of a kidney and a portion of the spleen were preserved in optimal cutting temperature (OCT) reagent and immediately frozen on dry ice and stored at -80˚C until sectioned. OCT frozen tissue was cut into 5 µm sections and mounted on slides. OCT slides were kept at -80˚C prior to staining.

Immunofluorescent staining

Immunofluorescent staining was done 5 µm sections from OCT-preserved tissue. Two sections were mounted on each histology slide, but separately stained. On day 1, Unstained sections were moved to room temperature, then fixed in cold reagent acetone, followed by three washes in 1x PBS. Tissue was isolated with a pap pen barrier (Vector Laboratories) and blocked with 10% normal goat serum (Life Technologies) prior to staining. Antibodies, noted in Table 2, were added to the sample and incubated overnight at RT in a humidified chamber. On day 2, slides were washed in 1xPBS, as described above, and a coverslip was applied using a 10% glycerol solution. Samples were stored for up to two weeks at 4˚C until imaged.

Periodic Acid Schiff (PAS)

Paraffin-embedded kidney sections were washed 3x in xylene for 5 minutes per wash, followed by 2x washes in 100% ethanol for 10 minutes per wash, then 2x washes in 95% ethanol for 10 minutes per wash, and finished with 2x washes in milliQ water for 5 minutes each. Tissue was then isolated on the slide using ImmEdge™ Pen (Vector Laboratories). RT PAS was added drop-wise to tissue and incubated for 5 minutes at RT, then washed 3x in milliQ water. Schiff reagent was then added to sections and incubated for 15 minutes, followed by rinsing in lukewarm tap water for 8 minutes, then washed 2x in milliQ water by dipping 5x. Tissue was then dipped in hematoxylin (Richard Allen) for 5 seconds, followed by washing 2x in milliQ water by dipping 5x, and run under tap

water for 2 minutes. The tissue was washed 2x in water for 5 minutes, followed by 4x 1 minute ethanol washes, 2 at 95% and 2 at 100%. Staining was finished with 2 xylene washes for 5 minutes each and a coverslip was applied using mounting medium (Richard Allan).

CD4 & CD8 Staining

This staining was done by Nina Dvorina. Paraffin-embedded kidney sections (5 µm) were chemically deparaffinized by incubating 2x in Clear Rite 3' (ThermoScientific Richard Allan). Tissue was then re-hydrated by washing 2x in Flex 100 (ThermoScientific Richard Allan), followed by washing 2x in Flex 95 (ThermoScientific Richard Allan), and finishing by rinsing the slides in tap water. Tissue was isolated on slides using a hydrophobic pen and ~100uL HBSS 2% FBS was applied to block tissue for 30 minutes, then removed. Anti-CD4 or Anti-CD8 antibodies (Abcam) were diluted 1:1500 in blocking solution and added to the tissue for incubation overnight at RT in a humidity chamber. The next day slides were washed with 1xPBS and rabbit horse-radish peroxidase (HRP—conjugated rabbit anti-rodent) (Biocare Medical RMR622H) was applied for 20 minutes. Slides were then washed in 1xPBS and stained using the DAB kit, used for chromogenic development of HRP, for one minute and then washed in water. Slides were then stained with hematoxylin 7211 (ThermoScientific) for one minute, followed by clarifier, and a bluing reagent. Slides were then washed in three rounds of ethanol at increasing concentrations (70%, 90%, and 95%) and finished with incubation in Clear Rite before mounting media was applied to finish the staining. Preliminary staining was done on a subset of samples. B6: n = 2, B6. TΔIFNAR: n = 2 B6.Nba2: n = 4, B6.Nba2.TΔIFNAR: n = 5.

Imaging

Imaging was done on either a Leica DMR upright microscope in the Cleveland Clinic Microscopy Core or an "All in One" BZ-X series Keyence microscope (Keyence, Osaka, Japan). Images were analyzed, colored, and quantified using Image Pro Plus 7 software (Media Cybernetics) or analyzed using the BZ-X Keyence Analysis software.

Scoring

H&E and PAS staining of kidney tissue was blindly scored by a renal pathologist: Dr. Jane Nguyen M.D. Kidneys were scored for mesangial and endothelium hypercellularity on a scale of 0-5. Area of 5–15 individual glomeruli per section for each mouse were averaged for individual glomerular area, as previously published[33].

Flow Cytometry

Cells were aliquoted into an uncoated 96-well plate, centrifuged, and suspension buffer, 1xPBS, was removed. Fc block (anti-CD16/anti-CD32 monoclonal antibodies, eBioscience) was diluted 1:200 in 1xPBS, and 50ul was added directly to the cells and incubated on ice for 30 minutes. Stain mix, containing fluorophore conjugated antibodies found in Table 2 diluted in 1xPBS were added directly to cells and incubated on ice in the dark for an additional 30 minutes. Dilutions ranged from 1:25 to 1:400, depending on the fluorophore used, and was optimized for each antibody. Post incubation, samples were quenched with 2x volumes 1xPBS, centrifuged, and buffer containing the antibody cocktail was removed. Cells that were only being stained for surface markers were suspended in 1% PFA in 1xPBS and transferred into microtiter tubes for flow cytometry and stored at 4˚C in the dark and run within 7 days. Cells that were being stained for intracellular markers were stained using the FOXP3+ intracellular staining buffers and done as previously described [33]. Briefly, samples were fixed and permeabilized for a

minimum of 30 min, although usually overnight. The next day, samples were washed in permeabilization buffer and incubated with Fc block as described above. Antibodies against intracellular proteins were diluted in permeabilization buffer and added to the cells for additional 30 min incubation, after which the cells were washed and transferred

Target	Clone	Company	Target	Clone	Company
Anti-Rabbit	Poly4064	ebioscience	IgG	cat#: 1032-05	Southern Biotech
B220	RA3-6B2	ebioscience	IgG	T862	Life Technologies
BCL2	Cat #51-15025X	BD Biosciences	IgG1	cat#: 1070-05	Southern Biotech
Bcl6	BCL-DWN	ebioscience	IgG1 (γ1)	A21124	Invitrogen
BIM	C35C3	Cell Signalling Technologies	IgG1κ	eBRG1	ebioscience
CD 127 / IL-7R	A7R34	ebioscience	IgG1κ	MOPC-21	Biolegend
CD117/CKIT	2B8	ebioscience	IgG2aκ	eBR2a	ebioscience
CD11b	M1/70	ebioscience	IgG2b	cat#: 1090-05	Southern Biotech
CD11c	N418	ebioscience	IgG2bκ	eB149/10H5	ebioscience
CD138	281-2	ebioscience	IgG2bκ	MPC-11	Biolegend
CD16/32	93	ebioscience	IgG2c	cat#: 1079-05	Southern Biotech
CD19	1D3	ebioscience	IgG2c	Cat# 1077-31	Southern Biotech
CD21/35	4E3	ebioscience	IgG3	cat#: 1100-05	Southern Biotech
CD22	OX-97	Biolegend	IgM	eB121-15F9	ebioscience
CD23	B3B4	ebioscience	IgM	11/41	ebioscience
CD25	PC61.5	ebioscience	IgM	cat#: 1020-05	Southern Biotech
CD3	145-2C11	ebioscience	IgM	1020-07	Southern Biotech
CD38	90	ebioscience	IL-10	JES5-16E3	ebioscience
CD4	GK1.5	ebioscience	IL-4	11B11	ebioscience
CD40	HM40-3	ebioscience	LY6C	AL-21	ebioscience
CD40L	MR1	ebioscience	Ly6C	AL-21	BD Biosciences
CD43	R2/60	ebioscience	LY6G	1A8	ebioscience
CD44	IM7	ebioscience	Ly6G	1A8	BD Biosciences
CD5	53-7.3	ebioscience	MHC-II	M5114.15.2	ebioscience
CD62L	MEL-14	ebioscience	MOMA-1	MOMA-1	Abcam
CD69	H1.2F3	ebioscience	PCDA1	927	ebioscience
CD8	53-6.7	ebioscience	PD-1	J43	ebioscience
CD86	GL1	ebioscience	RorYT	AFKJS-9	ebioscience
CD93	AA4.1	ebioscience	Sca-1	D7	ebioscience
Complement C'3	GC3-90F-Z	ICL Lab	Sca-1	D7	ebioscience
CXCR5	Cat:551960	BD Pharmigen	SigH	440c AF647	ebioscience
F4/80	BM8	ebioscience	SignR1	AF647	ebioscience
FoxP3	FJK-16s	ebioscience	Streptavidin	4317	ebioscience
GL-7	GL-7	ebioscience	Streptavidin	S11226	Invitrogen
Gr1	RB6-8C5	ebioscience	Tbet	eBio4B10	ebioscience
IgA	cat#: 1040-05	Southern Biotech	Ter119	119	ebioscience
IgD	11-26c	ebioscience			

Table 2: Antibodies utilized in various experiments are noted here by clone number, or catalog number if a clone number wasn't available. Conjugation of the antibody to fluorophores, biotin, or HRP is indicated by the column title. Antibodies conjugated to various Alexafluors are listed in the column of the fluorophores that they overlap with in excitation. Manufacturing company is noted on in the right hand column.

to microtiter tubes for immediate (<24 hours) flow cytometric analysis. All flow cytometry data was collected on a BD Fortessa Flow Cytometer in the Department of Inflammation and Immunity in the LRI. Data was analyzed using Flow Jo Analysis software. Cell populations were gated according to the strategy shown in Figure 3A for splenocytes and Figure 3B for bone marrow.

Flow Sort

Cells were stained in the same way as described in the flow cytometry section. Cells were suspended in 500uL 1xPBS. Populations were sorted based on surface marker expression by Jennifer Powers using the BD FACSARIA™. Populations were checked for purity by J. Powers prior to lab acquisition. Upon lab acquisition, cells were centrifuged, suspended in 1xPBS and counted using a Beckman-Coulter Cell Counter for further use in experiments.

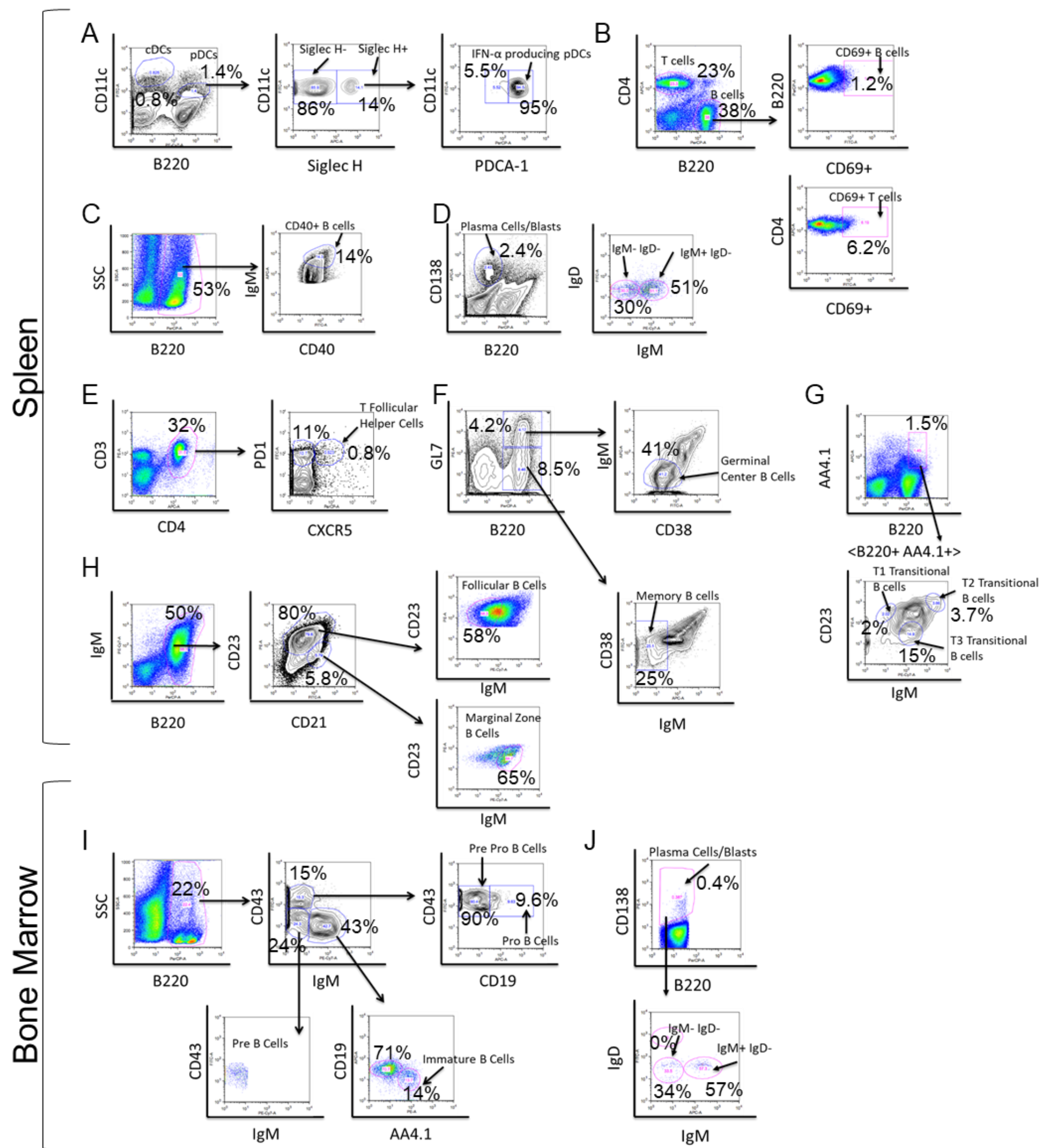

Figure 3: Gating strategy for splenocyte and bone marrow populations shown in figures. A) pDCs in were gated as CD11c+ B220+ and IFN-α producing pDCs were gated as SigH+ PDCA1+. cDCs were gated as B220- CD11c+ B) CD69+ activated cells were determined from B220+ B cells and CD4+ T cells. C) CD40+ activated B cells were gated as IgM+ CD40+ from B220+ B cells. D) Plasma blasts/cells were gated as B220 low CD138+ and then separated by IgD and IgM. E) T follicular helper cells were gated as PD1+ CXCR5+ from the CD3+ CD4+ T cell population. F) Germinal center cells were gated as B220+ GL7+ and IgM- CD38-, while memory cells were gated as B220+ GL7-, then CD38+ IgM-. G) B220+ AA4.1 + cells were separated into transitional B cell populations using IgM and CD23. H) B220+ IgM+ cells were separate into CD23+ CD21- follicular B cells and CD23- CD21+ marginal zone B cells. I) Bone marrow was separated into developmental subsets using B220, CD19, AA4.1, and IgM. J) Plasma blasts/cells were defined as B220 low CD138+, then separated by IgM and IgD expression. All subsets shown were first gated for live cells using FSC SSC pattern. This figure and figure legend was previously published by our lab [33].

Enzyme Linked Immunosorbent Assay (ELISA)

In-house ELISAs; Immunoglobulin and Chromatin

ELISAs measuring total IgM, total IgG and IgG subtypes, and anti-chromatin IgG were made in-house and run as previously published [33]. First, 96-well Immunlon 2HB plates flat bottom microtiter plates (ThermoScientific, Waltham, MA, USA) were coated with either unlabeled Ig or purified chromatin, diluted in 1xPBS, and incubated at 4˚C overnight. Plates were then washed 3 times with 1xPBS with 0.05% tween and blocked with 0.05% gelatin in 1xPBS and stored at 4˚C until used for up to two weeks. Samples were diluted in serum diluent (1xPBS with 0.05% tween, 0.05% Gelatin, 0.05% γ-globulin) to the concentration noted in Table 3 and applied to the pre-coated plate and incubated at RT for 90 minutes.

ELISA	Dilution	Sample Type
IgM	1:100,000	Serum
IgG	1:100,000	Serum
IgG1	1:50,000	Serum
IgG2b	1:100,000	Serum
IgG2c	1:100,000	Serum
IgG3	1:50,000	Serum
Anti-dsDNA	1:100	Serum
Anti-nRNP	1:100	Serum
Anti-Chromatin	1:300	Serum
NP-CGG21	1:10,000	Serum
NP-Ficoll	1:250,000	Serum
BAFF	15:100	Spleen Supernatent
IL-6	1:4	Spleen Supernatent
IL-10	1:4	Spleen Supernatent
IL-21	1:3	Spleen Supernatent

Table 3: Dilution of serum for various ELISA assays. Serum or spleen supernatant was diluted in the serum diluent buffer noted in each experiment.

Samples were removed, plates washed as above, and secondary HRP-conjugated antibodies specific for IgG, IgG subtypes, or IgM and diluted in 1xPBS was applied to the plate and incubated at RT for 90 minutes. The plate was washed as described above, and TMB substrate (1:1 ratio of 3, 3′, 5, 5′-Tetramethylbenzidin: H2O2 (ThermoScientific)) of was applied and incubated in the dark until the standards had developed. The reaction was quenched with sulfuric acid (Spectrum Chemical MGF CORP) and the optical density (OD) was measured at 450nm using a Victor 3 plate reader (Perkin Elmer).

Manufacturer Kit ELISAs;

Spleen supernatants were analyzed for cytokines according to manufacturer's instructions: IL-6 (R&D Biosystems, Minneapolis, MN, USA), IL-21 and BAFF (MyBiosource, San Diego, CA, USA). Spleen supernatant was diluted in serum diluent provided for each respective kit. Dilutions used are noted in Table 3.

Anti-dsDNA and anti-nRNP ANAs were measured using mouse anti-dsDNA IgG and anti-nRNP IgG ELISA kits (Alpha Diagnostics International, San Antonio, TX, USA) according to the manufacturer's instructions. Samples were diluted as noted in Table 3. IgG subtype dsDNA ELISAs were performed using the anti-dsDNA kit above, but developed using anti-IgG subtype specific-HRP antibodies (Southern Biotech) [33].

Immunizations

NP-CGG_{21} Immunization

Mice were immunized intraperitoneally with 1cc of NP-CGG_{21} mixed with complete Freund's adjuvant (CFA) and sterile 1xPBS. Mice were bled for serum the day prior to immunizations, 7 days post immunization, and harvested 14 days post immunization. The anti-NP antibody response was measured by ELISA. Immunlon 2HB plates were coated with 5ug/mL NP-5-BSA in PBS. Samples were diluted as noted in Table 3 and ELISA was developed with IgG_1-HRP (1:2000) (Southern Biotech).

NP-Ficoll Immunization

Mice were immunized intraperitoneally with 200uL of NP-Ficoll mixed with CFA and sterile 1xPBS. Mice were bled for serum the day prior to immunizations, 7 days post immunization, and harvested 14 days post immunization. The anti-NP antibody response was measured by ELISA. 2HB plates were coated with 5ug/mL NP-5-BSA in PBS.

Samples were diluted as noted in Table 3 and ELISA was developed with IgG-HRP (1:2000) (Southern Biotech).

ELISpot

Plasma Cells IgG Production

Primary splenocytes were FACs sorted for PCs (CD19 low CD138+). Sorted PCs were suspended into primary cell media and plated onto a multiscreen IP filter ELISpot plate (Millipore) that had been coated with unlabeled Ig (Southern Biotech) overnight and then blocked for 2 hours minimum with primary cell media at RT. After plating, cells were stimulated with recombinant mouse IFN-α in PBS with 0.1% BSA (Fisher Scientific) in primary cell media or primary cell media alone overnight. The next day, cells were removed, the plate was washed, incubated with an anti-IgG-HRP conjugate (1:2000) for 2 hours and developed with the Pierce™ TMB substrate kit (ThermoScientific) for 30 minutes. Developer was removed with running tap water and plates were dried overnight. Once dry, plates were imaged and analyzed using an ImmunoSpot Series 2 Analyzer (Cellular Technology Ltd.).

U-PLEX Mouse Biomarker Assay

Cytokine concentrations in the spleen supernatant of the TΔIFNAR mice and corresponding controls were quantified using a custom U-PLEX mouse biomarker assay from Mesoscale Discovery (MSD). The antibody and linkers were coupled, then the plate was coated, and undiluted spleen supernatant and calibrators applied, all according to manufacturer instructions. The plate was developed and the concentration measured using an MSD instrument.

Quantitative polymerase chain reaction (qPCR)

We utilized qPCR to measure expression of specific transcripts. RNA was isolated from samples using the RNeasy systems from Qiagen (Germantown, Maryland). RNA was used to make cDNA using qscript cDNA Supermix (QuantaBio Beverly, MA). Reactions were set up using Sybr Green (Sigma-Aldrich St. Louis, MO) and gene-specific primers (Integrated DNA Technologies, Coralville IA) and run using the StepOne™ Real-Time PCR Systems Instrument (Applied Biosciences). The primer sequences utilized can be found in Table 4.

Target Name	Forward Sequence 5'-3'	Reverse Sequence 5'-3'
Aicda	GCG GAC ATT TTT GAA ATG GTA	TTG GCC TAA GAC TTT GAG GG
Baff	CAG CGA CAC GCC GAC TAT AC	CCT CCA AGG CAT TTC CTC TTT T
Bcl2	TGA GTA CCT GAA CCG GCA TCT	GCA TCC CAG CCT CCG TTA T
Bcl6	CCT GAG GGA AGG CAA TAT CA	CGG CTG TTC AGG AAC TCT TC
Bclxl	TGG AGT AAA CTG GGG GTC GCA TCG	AGC CAC CGT CAT GCC CGT CAG G
Bim	CGG ATC GGA GAC GAG TTC	TTC CAG CCT CGC GGT AAT CA
Blimp	TAG ACT TCA CCG ATG AGG GG	GTA TGC TGC CAA CAA CAG CA
Ifi202	GGT CAT CTA CCA ACT CAG AAT	CTC TAG GAT GCC ACT GCT GTT G
Il10	CAG AGC CAC ATG CTC CTA GA	TGT CCA GCT GGT CCT TTG TT
IL-1β	CCC TGC AGC TGG AGA GTG TGG A	CTG AGC GAC CTG TCT TGG CCG
Il21	CGC CTC CTG ATT AGA CTT CG	TGG GTC TCC TTT TCT CAT ACG
Il6	ACA CAT GTT CTC TGG GAA ATC GT	AAG TGC ATC ATC GTT GTT CAT ACA
Irf4	GCC CAA CAA GCT AGA AAG	TCT CTG AGG GTC TGG AAA CT
Mx1	TTC CTG AAG AGG CGG CTT T	GGT TAA TCG GAG AAT TTG CCA A
Pan IFN-α	CTT CCA CAG GAT CAC TGT GTA CCT	TTC TGC TCT GAC CAC CTC CC
β-actin	TGG GAA TGG GTC AGA AGG AC	GGT CTC AAA CAT GAT CTG GG

Table 4: Sequences of real-time PCR primers. Primers were ordered from Integrated DNA Technology and suspended in RNAse free water and used at a concentration of 10uM.

Ex Vivo Experiments

Calcium Flux Assay

B cells were enriched from total splenocytes using a magnetic assisted cell sort (MACS) based CD43 depletion (Miltenyi Biotech). Enriched B cells (10 x 10^6 per mL) were applied a 1:1 ratio of Fluo-3: Pluronic Acid (Invitrogen both) and incubated at 37˚C for 45 minutes. Cells were then washed in calcium assay buffer (Phenol-red free DMEM, 1% BSA, 1% HEPES) and re-suspended in calcium assay buffer at a concentration of 1 million/ mL and aliquoted. Cell aliquots were kept at 37˚C in a water bath until the aliquot was utilized. Calcium flux was measured by flow cytometry. Flow cytometry data

was recorded for 30 seconds, the sample was removed, any stimulant used was added in the space of ~10 seconds, and the sample was placed back on the instrument to gather the flux data for the time remaining (3 minute total run time). Stimulants included; recombinant IFNαA (1000U/mL) (PBL Assay Science), IgM Fab fragment (0.6 µg/mL) (Jackson Immunoresearch), and ionomycin (ChemCruz®) as a positive control (1ug/mL). An unstimulated sample was included as a negative control.

T Helper Cell Skewing

Prior to sacrificing mice, a 96 well tissue culture (TC) plate was coated with 100uL/well of 1:1000 α-CD3 and α-CD28 antibodies (eBioscience, San Diego CA) diluted in 1x sterile PBS for 2-4 hours at 37˚C. RBCs were removed from spleen single cells using ACK, as above, and cells were counted and re-suspended for magnetic cell sorting of Naïve CD4 T cells. Isolation was done using the Naïve CD4 T cell isolation kit from Miltenyi (Bergisch Gladbach, Germany / Auburn, CA, USA, Catalog #130-104-453) according to manufacturer's instructions. Post isolation, Naïve CD4 T cells were suspended as 200 million cells per mL, twice the final concentration, in primary cell media. The α-CD3 and α-CD28 coating was removed from the TC plate and 100uL, 20 million cells, were plated per well. Primary cell media containing skewing cytokines was added, 100uL per well, bringing the final concentration of Naïve T cells to 100 million per mL. Cells were incubated in media for three days at 37˚C to allow time for transition. TH1 skewing media contained 10ug/mL anti-IL-4 antibody and 10 ng/mL recombinant IL-12. TH2 skewing media contained 20 µg/mL anti-mIFNγ antibody, 20 µg/mL anti-mIL-12 antibody, and 4 ng/mL recombinant IL-4. Samples that received recombinant IFNαA received 1000 U/mL.

After incubation, cells were harvested from the plate and prepared for staining. Using the staining protocol above, cells were incubated with Fc block and stained for surface markers using anti-CD3, anti-CD4, and anti-CD8 antibodies (see Table 2, above). Cells were then permeabilized, as previously described, and stained intracellularly for Tbet and IFN-γ, Gata3 and IL-4, or with isotype controls IgG1k–PE and IgG2b–APC or IgG2b-PE and IgG2b–APC respectively. Flow cytometry was run on samples.

Statistics

Statistics were calculated using Prism Graph Pad software. Student's unpaired T-test with Welch's correction was utilized when comparing two groups of unpaired samples. Two-way ANOVA comparing time and genotype was used for longitudinal bleeds in nine month B6.Nba2.BΔIFNAR mice. Specific statistical analyses for each experiment are noted in the figure legend. Statistical significance are given as: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

CHAPTER 3: BΔIFNAR

Hypothesis and rationale

As one of the predominant challenges with SLE is heterogenic pathogenic presentation, it would be ideal to understand the pathogeneses that are most predominant in the patient population. Approximately 98% of SLE patients have a positive ANA, making ANA reduction a critical measurement of disease [268]. Antibodies are produced by the plasma cells and the plasma cells develop primarily as a result of B cells going through the germinal center reaction [67]. A significant amount of SLE presentations are characterized by the B cell dysfunction such as splenomegaly, lymphadenopathy, and immune complex deposition. While SLE can be managed using general immunosuppression, the targeted treatments commonly used in SLE are B cell based.

Rituximab is used off label to deplete B cells [269] and Belimumab is used to prevent the activation of B cells by binding to BAFF, a B cell activating factor [270]. As IFN-I can exacerbate the same B cell dysregulation seen in SLE, including plasma cell differentiation, our rationale was that increased levels of IFN-I stimulation were acting on the B cells to perpetuate their dysregulation in disease [60,271].

To study the direct pathogenic impact of IFN-I stimulated B cells, we utilized a B cell specific knockout in a spontaneous lupus model, driven by the *Nba2* locus. *Nba2*-driven lupus like disease is driven by increased IFN-I signaling [19,35]. Many of the *Nba2*-driven disease presentations are associated with B cells, including spontaneous germinal center formation, splenomegaly, activation of B cells, and increased populations of GC B cells, PCs, and Memory B cells [21,35].

Knowing that B6.Nba2 lupus-like disease is driven by IFN-I and has many B cell associated disease presentations that could be a response to IFN-I led to our following hypothesis. **We hypothesized that the B cell-hyperactivity and autoantibody production in B6.Nba2 mice is driven by IFN-I signaling through IFNAR expression by B cells.**

Results: Disease Phenotype

In the following sections I will present data obtained from analyzing four month old B6.Nba2.BΔIFNAR mice. I will also present a selection of unpublished data from nine month old mice, to illustrate relevant differences between early and late disease. Data from the four month old mice was recently published in Frontiers in Immunology (Keller et al [33]). Figures and figure legends that were included in this publication and appear

without further alterations will be noted in the figure legend with the original citation [33].

B cells in BΔIFNAR mice do not respond to IFNα

B6.BΔIFNAR and B6.Nba2.BΔIFNAR mice were identified by genotyping as IFNAR$^{flx/flx}$ and MB1-cre+ (Figure 4A). For all studies, a mix of IFNAR$^{flx/flx}$ MB1-cre^{-}, IFNAR$^{+/+}$ MB1-cre^{+} and IFNAR$^{+/+}$ MB1-cre^{-} littermates were used as controls. There were no lupus-like phenotype in any of these mice. To ensure that B cells were functional IFNAR knockouts, we measured the upregulation of Ifit2, an IFNα inducible protein, by flow cytometry. B cells from BΔIFNAR mice were unable to produce Ifit2 in response to

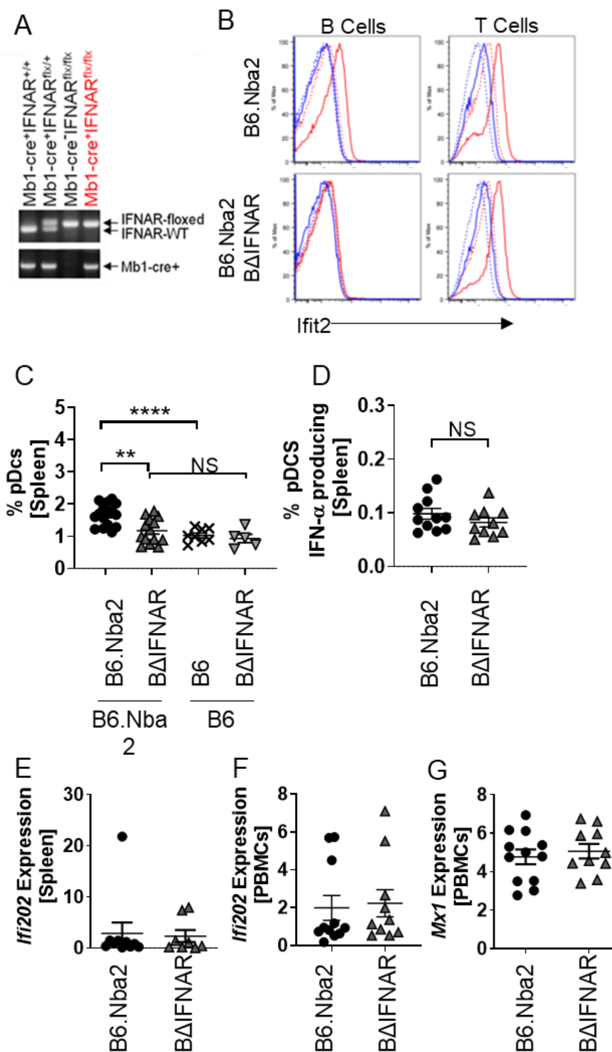

Figure 4: B cells from B6.Nba2.BΔIFNAR mice do not respond to recombinant IFN-α *in vitro*. A) Genotyping by PCR shows the presence of Mb1-cre and floxed IFNAR. B) Primary splenocytes were isolated and incubated overnight, with or without recombinant mouse IFN-αA. Induction of Ifit2 protein was determined in T and B cell populations using flow cytometry. Data shown are representative of four independent analyses. Blue stippled line: No stimulation, control stain; Blue solid line: IFN-α stimulation, control stain; Red stippled line: No stimulation, Ifit2 stained; Red solid line: IFN-α stimulation, Ifit2 stain. C-D) Splenic populations of pDCs (C) and SigH+ pDCs (D) were quantified in B6.Nba2 (n = 11-17), B6.Nba2.BΔIFNAR (n = 10-14), B6 (n = 8) and B6.BΔIFNAR (n = 5). E-G) Expression of Type-I interferon-induced transcripts was quantified using RT PCR for *Ifi202* (E-F) and *Mx1* (G) in the spleen and peripheral blood mononuclear cells (PBMCs), respectively. Each symbol represents one mouse and data are shown as Mean ± SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction. This figure and figure legend was previously published by our lab [33].

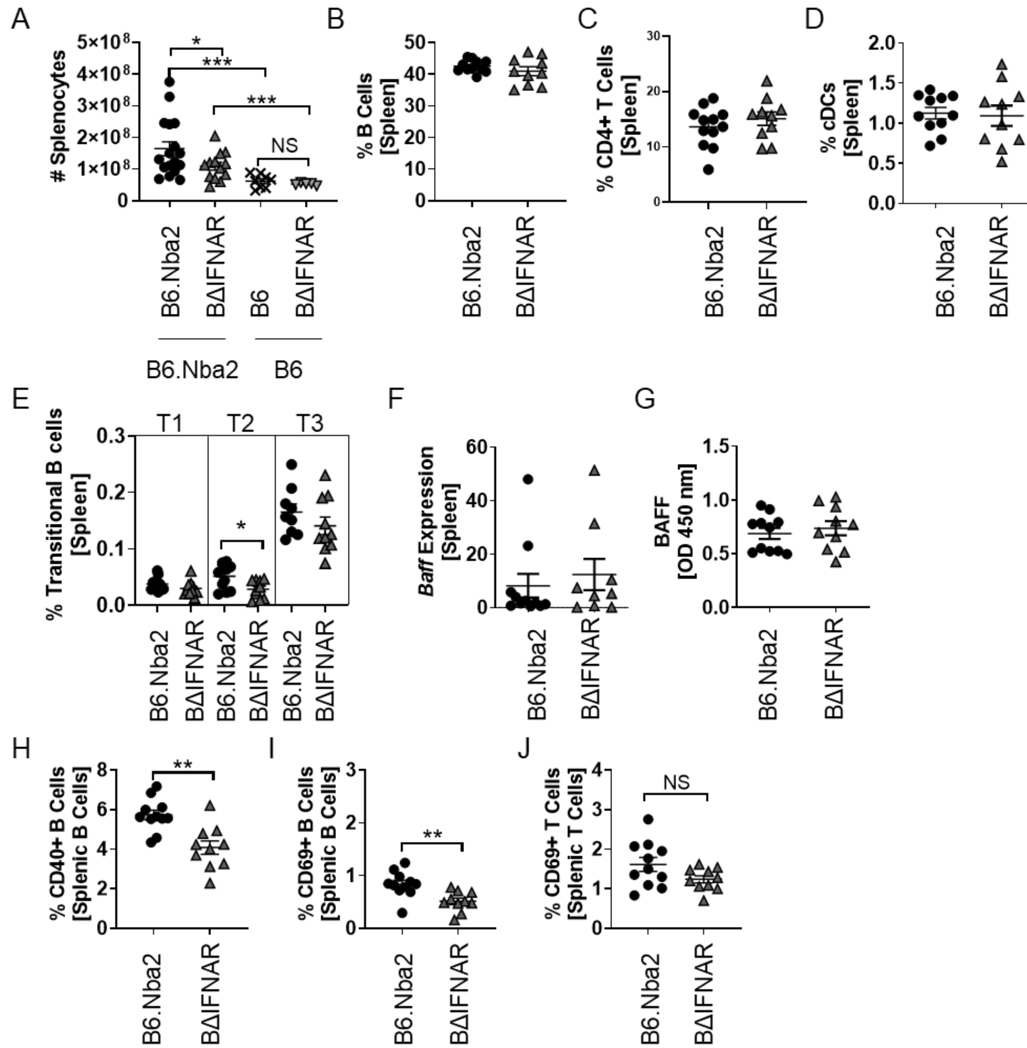

Figure 5: B6.Nba2.BΔIFNAR mice do not develop splenomegaly and display reduced populations of activated B cells. A) Splenomegaly was measured by splenocyte count. B-E) Percentages of total splenic B cells (B), T cells (C), cDCs (D), and Transitional B cell subsets (E) were quantified using flow cytometry. F-G) Expression of *Baff* was quantified using RT-PCR and splenic secreted Baff levels were measured using an ELISA. H-J) Populations of CD40+ B cells (H) and CD69+ activated B cells (I) and T cells (J) in the spleen were quantified using flow cytometry. Each symbol represents one mouse and data are shown as Mean ± SEM. B6.Nba2: n = 11-17, B6.Nba2.BΔIFNAR: n = 10-13, B6: n=8, B6.BΔIFNAR: n=5.* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction. This figure and figure legend was previously published by our lab [33].

IFNα (Figure 4B). While B6.Nba2.BΔIFNAR mice did have a significantly reduced population of pDCs compared to B6.Nba2 mice (Figure 4C), there was no difference in IFNα-producing pDCs (SiglecH+ PDCA1+) in the lupus-prone mice (Figure 4D). Real-

time (RT) PCR further confirmed that the lupus prone mice had similar levels of IFNα, as there was no difference in measured ISGs, *Ifi202* and *Mx1*, in either spleen cells or peripheral blood mononuclear cells (Figure 4 E-G).

Splenomegaly is significantly decreased in 4 month old B6.Nba2.BΔIFNAR mice

Splenomegaly is a common indicator of autoimmunity in mouse models of lupus. We observed a significant decrease in splenomegaly in B-cell [33] (Figure 5A). Due to this significant difference in splenomegaly, we analyzed major spleen subsets further by percentage, instead of cell number, to appropriately exclude differences that were only significant due to the difference in splenomegaly. Spleen composition between B6.Nba2 and B6.Nba2.BΔIFNAR mice did not differ in populations of total T Cells, B cells, Macrophages, cDCs or neutrophils [33] (Figure 5 B-D, and Figure 6 A-D). We noticed no compensatory changes in transitional B cells and no changes in BAFF expression or secretion in B6.Nba2.BΔIFNAR mice (Figure 5E-G).

Activation of B cells is significantly decreased in B6.Nba2.BΔIFNAR mice

We found that B6.Nba2.BΔIFNAR mice had significant reductions in both CD69+ and CD40+ activated B cell populations as determined by flow cytometry [33] (Figure 5H-I).

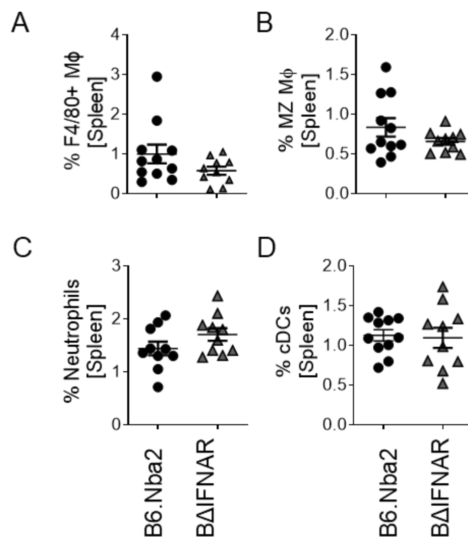

Figure 6: Populations of cDCs, macrophages, and neutrophils are unaltered in B6.Nba2.BΔIFNAR mice. Splenic populations of F4/80+ and marginal zone macrophages were quantified using flow cytometry by expression of F4/80 and SignR1 respectively(A-B). Splenic populations of neutrophils and cDCs were quantified in the same manner, using Ly6G to identify neutrophils and CD11c for cDCs (C-D). Each symbol represents one mouse and data are shown as Mean ± SEM. B6.Nba2: n = 11-17, B6.Nba2.BΔIFNAR: n = 10-13, B6: n=8, B6.BΔIFNAR: n=5.* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction.

As expected, we saw no difference in the activation of T cells, meaning that the decrease in activated B cells was not a result of a general decrease in lymphocyte activation (Figure 5J) [33]. As the MB1 cre driver appears in cells expressing the alpha chain of the B cell receptor (BCR), I additionally verified that the activation change was not a result of our model affecting BCR signaling. Calcium flux was measured by flow cytometry in response to stimulants and splenocytes from both B6.Nba2.BΔIFNAR and B6.Nba2 mice responded equivalently to the positive control stimuli: ionomycin or IgM (Figure 7A-B). Furthermore, calcium flux was not affected by the presence of recombinant IFNαA (Figure 7C-D), showing that any changes in B cell activation were unrelated to BCR signaling.

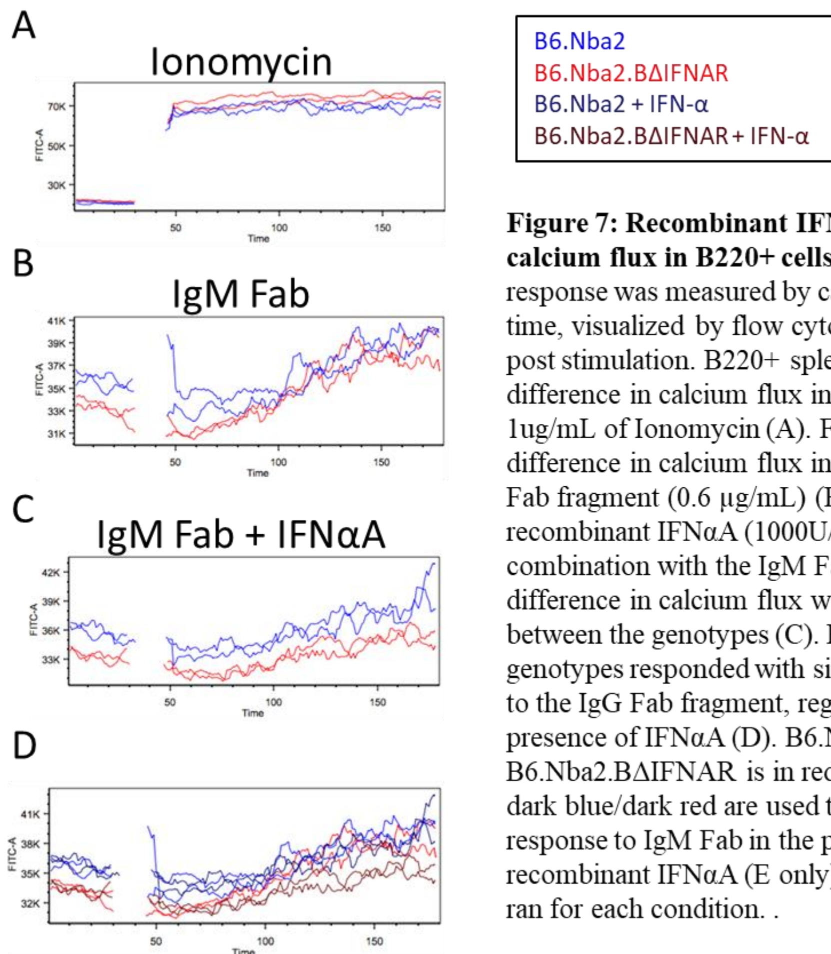

Figure 7: Recombinant IFNαA does not affect calcium flux in B220+ cells. B cell receptor response was measured by calcium flux over time, visualized by flow cytometry prior to and post stimulation. B220+ splenocytes showed no difference in calcium flux in response to 1ug/mL of Ionomycin (A). Furthermore, no difference in calcium flux in response to an IgM Fab fragment (0.6 µg/mL) (B). When recombinant IFNαA (1000U/mL) was added in combination with the IgM Fab fragment, no difference in calcium flux was observed between the genotypes (C). B cells from both genotypes responded with similar calcium flux to the IgG Fab fragment, regardless of the presence of IFNαA (D). B6.Nba2 is in blue, B6.Nba2.BΔIFNAR is in red (all figures), and dark blue/dark red are used to represent the response to IgM Fab in the presence of recombinant IFNαA (E only). Duplicates were ran for each condition. .

B6.Nba2.BΔIFNAR mice have reduced populations of splenic GC B cells

Consistent with our hypothesis we found significant decreases in the populations of splenic GC B cells as quantified using flow cytometry [33] (Figure 8E). This decrease was not due to altered B cell development in the bone marrow [33] (Figure 9A). Surprisingly, while we found a significant reduction in the population of germinal center B cells in B6.Nba2.BΔIFNAR mice, this reduction did not alter the average number or area of the germinal centers as quantified through IHC staining of spleen sections [33] (Figure 8F-G).

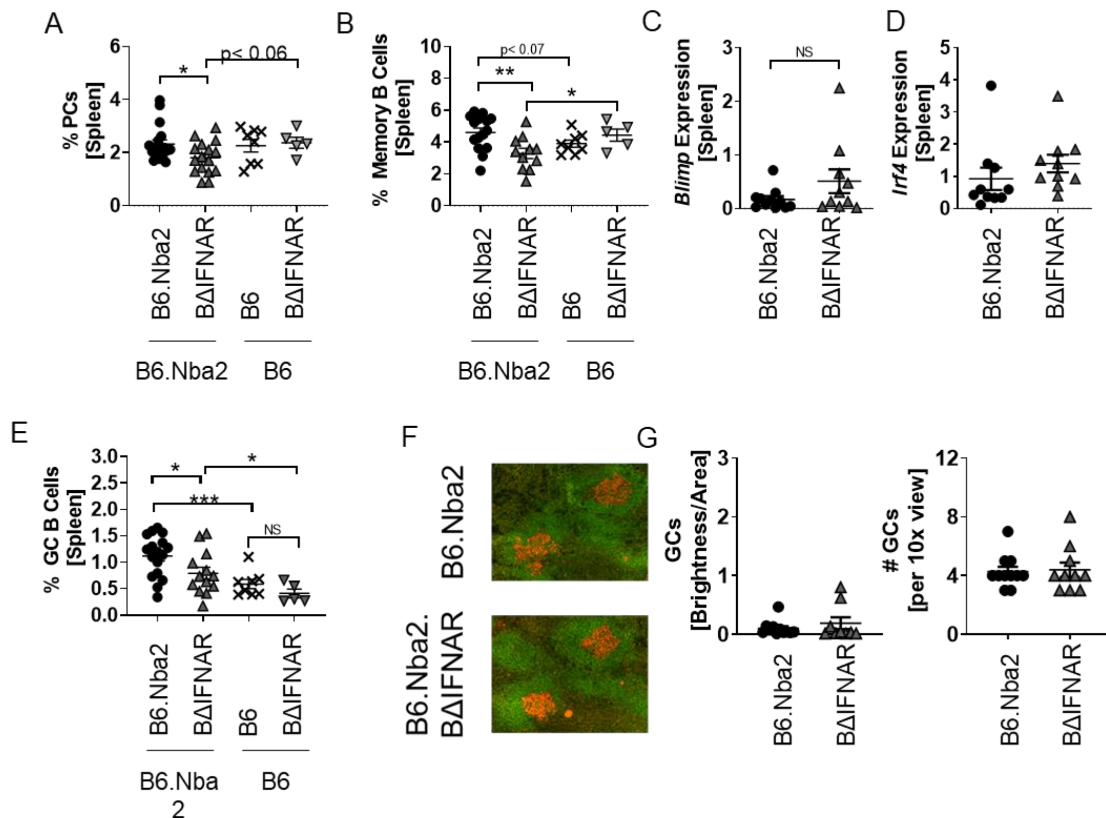

Figure 8: Antibody-secreting cells are reduced in spleens from B6.Nba2 BΔIFNAR mice. A-B) Splenic populations of antibody-producing PB/PCs (CD138+ B220low)(A) and memory B cells (B220+CD38+GL7-IgM-)(B) were measured by flow cytometry. C-D) Splenic transcripts of *Blimp1* (C) and *Irf4* (D) were measured by RT PCR. E) The splenic GC B cells (B220+ GL-7+IgM-) were quantified using flow cytometry. F) Immunohistochemistry staining for GCs using B220 (Green) and GL-7 (Red) was imaged. Representative images are shown. G) Mean GC brightness/area was measured and numbers of GCs in a 10x view were enumerated from stains shown in F. B6.Nba2: n = 11-17, B6.Nba2.BΔIFNAR: n = 10-14, B6: n = 8 (B6), B6. BΔIFNAR: n = 5. Each symbol represents one mouse and data are shown as Mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction. NS: not statistical. . This figure and figure legend was previously published by our lab [33].

B6.Nba2.BΔIFNAR mice have decreased PC and Memory B Cell populations

Due to the significant decrease in the GC B cell population, we quantified the PC and Memory B cell populations that develop via the germinal center reaction using flow cytometry. We found significant decreases in both the Memory B cell and PC populations [33] (Figure 8A-B). This decrease was not a result of increased homing of PCs to the bone marrow, as the population of PCs in the bone marrow remained unchanged [33] (Figure 9B). We assessed if the significant decrease was driven by changes in plasma cell differentiation, by quantifying transcription factors *Blimp* and *Irf4*, both known to be required for plasma cell differentiation [272–274]. We found differences in neither Blimp nor Irf4 in between B6.Nba2 and B6.Nba2.BΔIFNAR mice [33] (Figure 8C-D). This data allowed us to conclude that IFN-I stimulation of B cells was not altering the differentiation process of PCs and Memory B cells produced in the germinal centers.

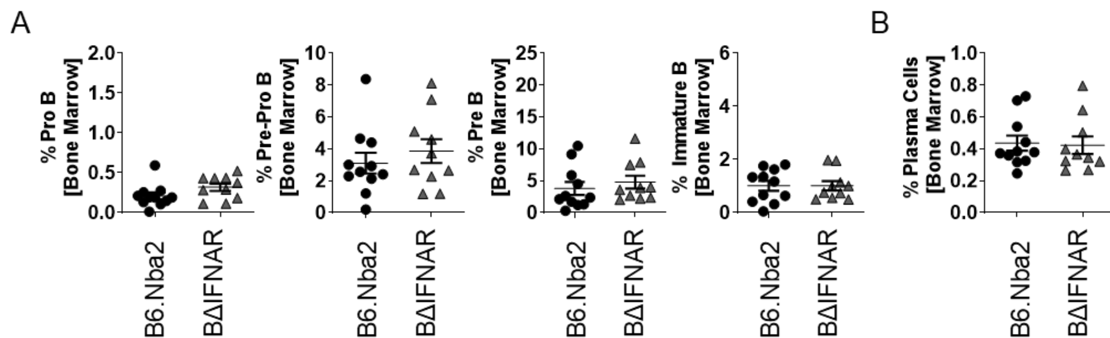

Figure 9: B cell development and bone marrow populations are unchanged on B6.Nba2.BΔIFNAR mice. Flow cytometry was done using the bone marrow from one femur and one tibia per mouse for cell samples. A) B cell development in the bone marrow was analyzed using flow cytometry and percentages of pro B cells, pre-pro B cells pre B cells, and immature B cells were quantified. B) Plasma cells were gated as B220 low CD138+. Each symbol represents one mouse and data are shown as Mean ± SEM. n = 11 (B6.Nba2); n = 10 (B6.Nba2.BΔIFNAR) * $p < 0.05$; ** $p < 0.01$; Student's unpaired t-test with Welch's correction. NS: not statistically significant. This figure and figure legend was previously published by our lab [33].

Reduction of GC B cells in B6.Nba2.BΔIFNAR mice is not accompanied by decreased TFH cells

As the TFH cells interact heavily with the GC B cells during the GC reaction that leads to the production of Memory B cells and PCs we wanted to determine if the TFH cell population was also altered. No significant difference was found in the size of the TFH cell population in the spleens of B6.Nba2 and B6.Nba2.BΔIFNAR mice (Figure 10A). We assessed the cytokine production capacity of the TFH cells by measuring transcript levels of *Il21*, *Il6,* and *Il1β* in the spleen, as well as spontaneously secreted cytokines from total splenocytes, and found that while splenic *Il21* transcripts were elevated in B6.Nba2.BΔIFNAR mice, there was no significant change in the levels of secreted IL-21 or IL-6 (Figure 10B-D).

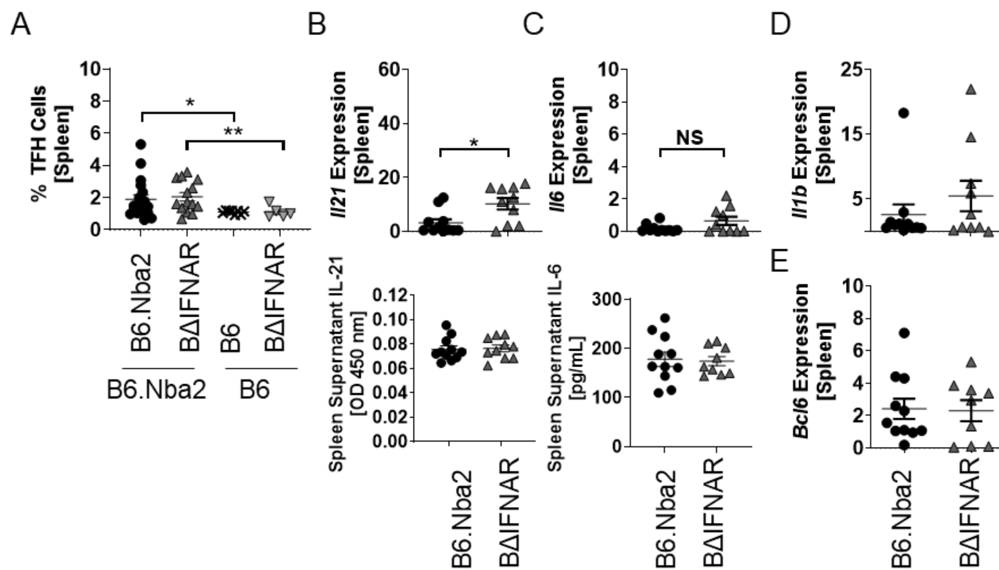

Figure 10: T follicular helper cell populations are not altered in B6.Nba2.BΔIFNAR mice. A) Populations of TFH cells (CD3+ CD4+ PD1+ CXCR5+) were quantified using flow cytometry. B-C) Transcripts and secreted splenic levels of IL-21 and IL-6were measured. D-E) Splenic levels of *Il1b* (D) and *Bcl6* (E) were measured using RT PCR. Each symbol represents one mouse and data are shown as Mean ± SEM. B6.Nba2: n = 11, B6.Nba2.BΔIFNAR: n = 10. * $p < 0.05$; ** $p < 0.01$; Student's unpaired t-test with Welch's correction. NS: not statistically significant. . This figure and figure legend was previously published by our lab [33].

Finally, *Bcl6* transcripts, expressed in GC B cells and the master transcription factor for TFH cells, was quantified and no significant differences were found between B6.Nba2 and B6.Nba2.BΔIFNAR mice (Figure 10E). It should be noted we observed no changes in other large populations of splenic B cells, in particular the MZ B cells and the follicular B cells (Figure 11A-B). Therefore the significant contribution of IFNα-stimulated splenic B cells to lupus-like disease progression was largely limited to the germinal center reaction.

GC B cells from B6.Nba2.BΔIFNAR mice have decreased expression of anti-apoptotic factors

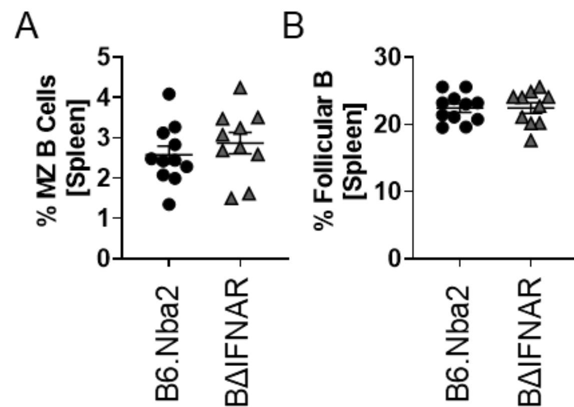

Figure 11: Populations of splenic Marginal Zone B cells and Follicular B cells are unchanged in B6.BΔIFNAR mice. Populations of marginal zone (B220+ CD23 intermediate CD21+ IgM+ and follicular B (B220+ CD21 low CD23+ IgM low) cells were quantified using flow cytometry. . This figure and figure legend was previously published by our lab [33].

IFN-I has been shown to contribute to increased survival in T cells [275] and lymphocytes [276]. To determine if the B6.Nba2.BΔIFNAR mice had a significant reduction in PCs and Memory B cells due to decreased survival, as we measured expression of the anti-apoptotic marker Bcl2. We saw no change in total splenic expression of *Bcl2* (Figure 12A) [33], so when we repeated the analysis using FACS sorted GC B cells, We quantified *Bim, Bclxl, and Bcl2* in populations of sorted GC B cells and PCs. Both *Bclxl* and *Bcl2* are anti-apoptotic[277,278], and *Bcl2* in particular has been reported to be upregulated in response to IFNα [276,279] *Bim* is known to be pro-apoptotic[277,278]. We found that GCs from B6.Nba2.BΔIFNAR mice had

significantly reduced *Bcl2* transcripts, and while not significant, these cells also showed a trend in decreased *Bclxl* and a trend towards increased pro-apoptotic *Bim* [33] (Figure 12B). PCs did not show the same significant difference in *Bcl2* and presented with less visible trends in *Bclxl* or *Bim* expression [33] (Figure 12C). We therefore concluded that the predominant effect of IFN-I B cell stimulation is increased survival of GC B cells, leading to increased GC cell populations and increased output of PCs and Memory B cells that develop through the GC reaction.

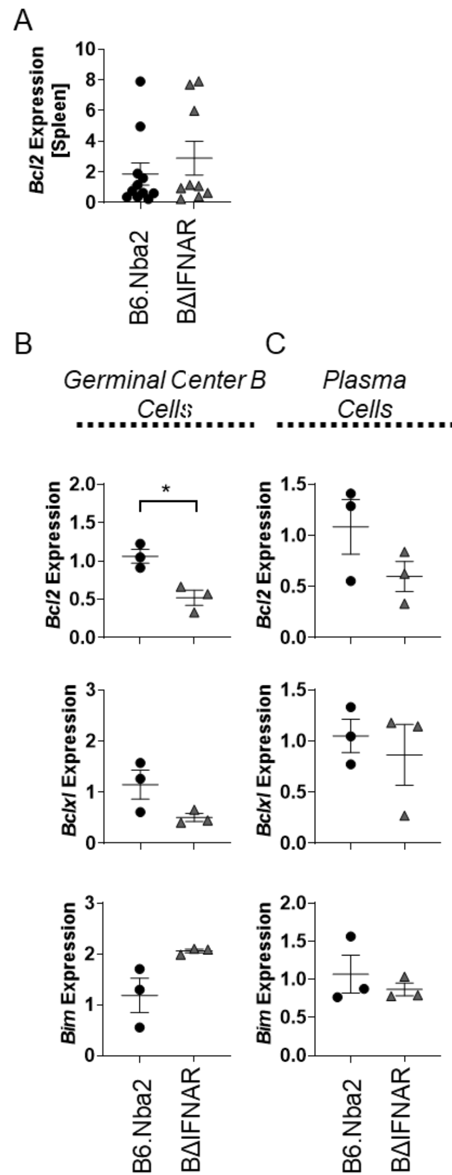

Figure 12: Bcl2 expression is decreased in GC B cells B6.Nba2.BΔIFNAR mice. Levels of *Bcl2* transcripts were measured in total spleen (A). Transcript levels of *Bcl2*, *Bclxl* and *Bim* were measured on sorted GC B cells (B) and sorted PB/PCs (C). Each symbol represents one mouse and data are shown as Mean ± SEM. B6.Nba2: n = 3, B6.Nba2.BΔIFNAR: n = 3. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction. . This figure and figure legend was previously published by our lab [33].

B6.Nba2.BΔIFNAR mice have reduced serum ANAs, particularly IgG_{2C} ANAs

As ANAs are a significant hallmark of SLE and B6.Nba2 lupus like disease in mice and because we saw a significant decrease in the antibody-producing PC population, we next quantified total immunoglobulin production as well as specific ANAs to determine if the PC population of B6.Nba2.BΔIFNAR mice differed in antibody production [67].

We found no significant difference in total levels of IgG or IgM as quantified by ELISA (Figure 13A-B) [33]. This was not due to a skewing of IgG subtypes, as all total IgG subtypes were unchanged [33] (Figure 13F). However, B6.Nba2.BΔIFNAR mice did have significantly decreased levels of anti-dsDNA, anti-Chromatin, and anti-nRNP IgG and were therefor protected from the level of ANA response commonly found in B6.Nba2 lupus-like disease (Figure 13C-E) [33]. In order to determine if a particular IgG subtype was impacted, we quantified antibodies against dsDNA and nRNP by IgG subtype via ELISA. We found that the anti-dsDNA IgG2c antibodies were significantly reduced and that anti-nRNP IgG2c antibodies were trending towards a reduction in B6.Nba2.BΔIFNAR mice (Figure 13G-H) [33].

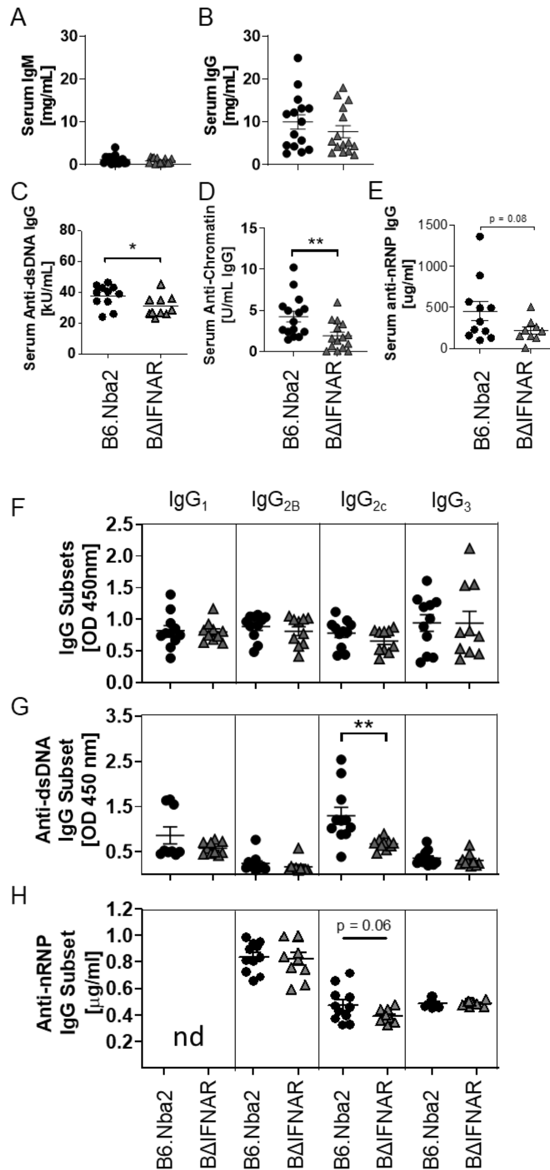

Figure 13: B6.Nba2.BΔIFNAR mice develop reduced ANA levels. Serum levels of antibodies were measured by ELISA in B6.Nba2 (n = 11-15) and B6.Nba2.BΔIFNAR (n = 10-15): IgM (A), IgG (B), anti-dsDNA IgG (C), anti-chromatin IgG (D), total serum IgG subtypes (IgG1, IgG2b, IgG2c, IgG3)(E) and anti-dsDNA IgG subsets (F). Each symbol represents one mouse and data are shown as Mean ± SEM. * p < 0.05; ** p < 0.01. Student T test with Welch's correction. This figure and figure legend was previously published by our lab [33].

B6.Nba2.BΔIFNAR mice are not protected from lupus-like glomerular disease

B6.Nba2 lupus-like disease has specific kidney presentations, in particular mesangial cell proliferation, immune

cell infiltration into the glomeruli, and immune complexes deposit in the glomeruli, where complement C3 fixes to them. Based on the reduction in autoantibodies in

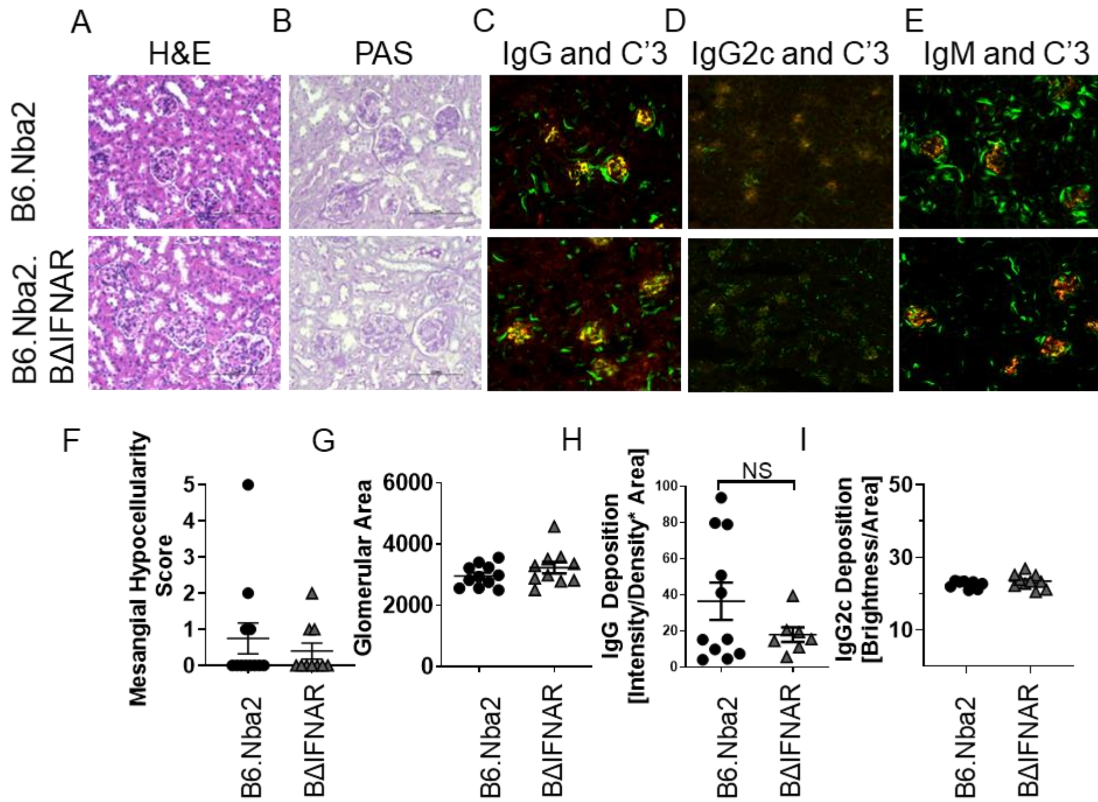

Figure 14: B6.Nba2.BΔIFNAR mice are not protected from glomerulonephritis or immune complex deposition. A) Kidneys from B6.Nba2 and B6.Nba2.BΔIFNAR mice were stained with hematoxylin/eosin (H&E). B) Kidneys from B6.Nba2 and B6.Nba2.BΔIFNAR mice were stained with periodic acid Schiff (PAS). C) Kidneys from B6.Nba2 and B6.Nba2.BΔIFNAR mice were stained with anti-IgG (Red)/anti-C'3 (Green). D) Kidneys from B6.Nba2 and B6.Nba2.BΔIFNAR mice were stained with anti-IgG2c (Red)/anti-C'3 (Green). E) Kidneys from B6.Nba2 and B6.Nba2.BΔIFNAR mice were stained with anti-IgM (Red)/anti-C'3 (Green). Stains were used for detection of renal morphology, glomerulonephritis, immune complex deposition and complement fixation. F) Kidneys were scored using both H&E and PAS stains by a blinded pathologist for mesangial hypercellularity. G) Glomerular area was measured and calculated using the H&E staining. H-I) IgG and IgG2c deposition were measured using Image Pro Software and Keyence software respectively. Two sections, representing > 10 glomeruli were analyzed per mouse for each evaluation. Each symbol represents the average score per mouse and data are shown as Mean ± SEM. n = 11 (B6.Nba2); n = 10 (B6.Nba2.BΔIFNAR). * $p < 0.05$, Student's unpaired t-test with Welch's correction. This figure and figure legend, without E, was previously published by our lab [33].

B6.Nba2.BΔIFNAR mice, we hypothesized that the kidneys may reflect the decrease and present with reduced immune cell infiltration and reduced immune complex deposition. H&E and PAS stains were performed on kidney sections and no difference was visualized (Figure 14A-B) [33]. Quantification of glomerular area and scoring of

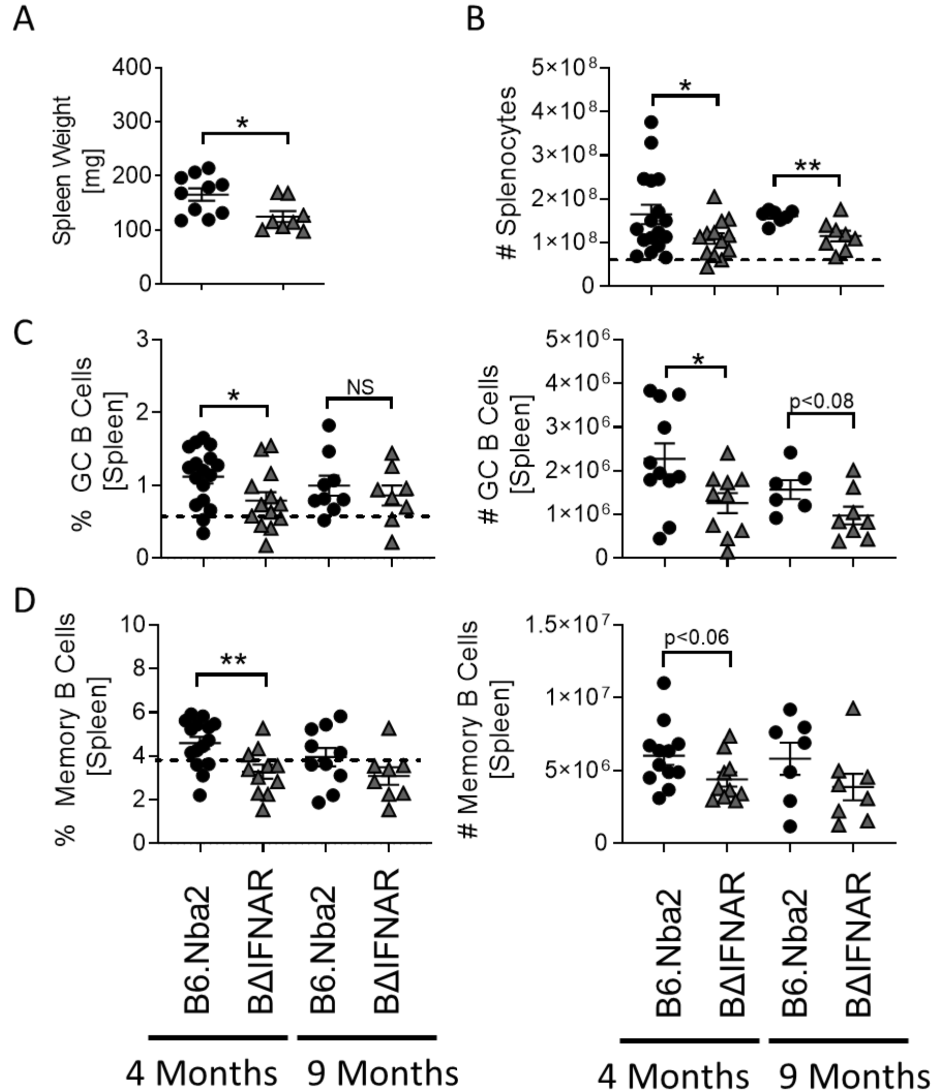

Figure 15: Older B6.Nba2.BΔIFNAR mice retain protection from splenomegaly but not decreased GC B cell populations. Splenomegaly was quantified in 9 month old mice by weight and splenocyte count (A,B). Flow cytometry was used to quantify percentages, and cell count was used to calculate total numbers of GC B cells and Memory B cells (C,D). 4 month old mice: n = 11 (B6.Nba2); n = 10 (B6.Nba2.BΔIFNAR). 9 month old mice: B6.Nba2 (n = 10) and B6.Nba2.BΔIFNAR (n = 8). Each symbol represents one mouse and data are shown as Mean ± SEM. * $p < 0.05$; ** $p < 0.01$. Student T test with Welch's correction.

mesangial hypercellularity, done in a blinded fashion by a renal pathologist, further confirmed this (Figure 14F-G). Immune complex deposition in the glomeruli was determined by immunofluorescent staining for IgG and IgM (red) in combination with complement C3 (green) and no difference in glomerular deposition or overlap (yellow) was seen (Figure 14C, E). We hypothesized that as the IgG_{2C} auto-antibodies were particularly decreased, but total IgG and IgG_{2C} levels were not, that we would see a specific decrease in deposited IgG_{2C} in the kidneys. However, no difference in IgG2C (red) and complement C3 staining was observed (Figure 14D). The lack of difference in IgG and IgG2C glomerular deposition was confirmed by fluorescent quantification (Figure 14H-I).

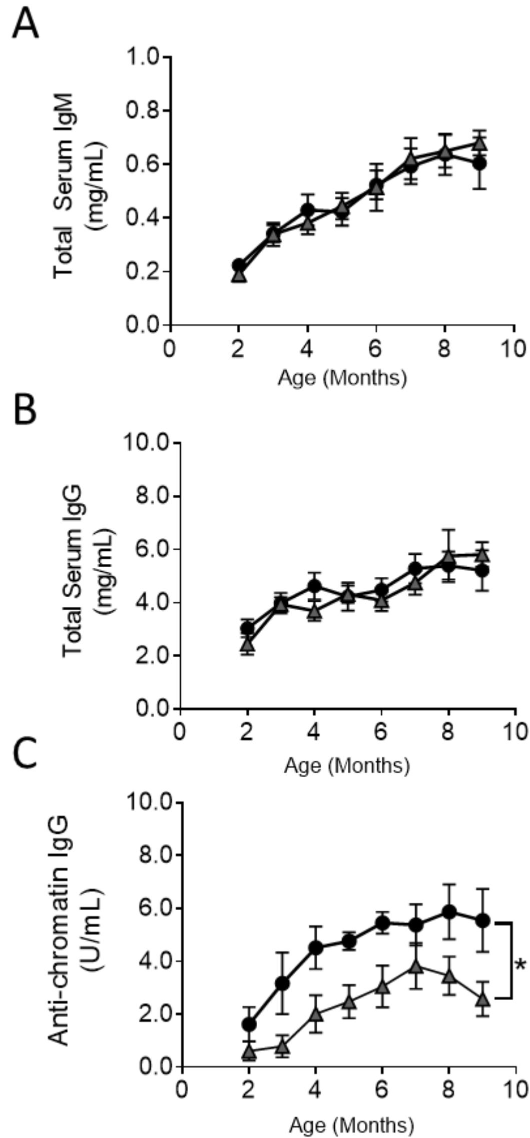

Figure 16: Older B6.Nba2.BΔIFNAR mice retain protection from disease with reduced serum Anti-Chromatin. Monthly serum levels of IgM, IgG, and anti-chromatin IgG were measured by ELISA in 9 month old B6.Nba2 (n = 10) and B6.Nba2.BΔIFNAR (n = 8) (A-C). Two-way ANOVA comparing time and genotype.

Type I IFN stimulated B cells have a larger pathogenic contribution early in disease

We harvested a cohort of late disease mice at 9 months of age, all on the B6.Nba2 background to assess disease

presentation. These older mice retained significantly reduced splenomegaly (Figure 15A-B) and maintained significantly lower levels of anti-chromatin, with no change in total IgG or IgM (Figure 16A-C). However, B6.Nba2.BΔIFNAR mice at nine months of age no longer showed reductions in the GC reaction-associated B cell populations of GC B cells and Memory B cells (Figure 15C, D). Similarly to the 4 month old mice, the 9 month mice were also not protected from immune complex deposition in the glomeruli and presented with levels similar to age-matched B6.Nba2 lupus mice (Figure 17A, B). These findings indicate that the pathogenicity of IFN-I stimulated B cells plays a larger role in earlier disease progression, but have less pathogenic contribute to later disease.

Previous unpublished data from our lab confirms the finding that the pathogenic effect of IFN-I is greater earlier in disease. Utilizing a BDCA2 linked diphtheria toxin receptor (DTR) transgenic model, pDCs were depleted from B6.Nba2 mice at various time points in disease (Manuscript in preparation Davison et al and [61]). When pDCs were depleted from younger mice, disease progression plateaued for the depletion period. However, when older mice underwent pDC depletion the disease progression was significantly less impacted. This data, combined with the finding that 9 month old B6.Nba2.BΔIFNAR mice do not present with the significantly decreased GC-related B cell populations seen

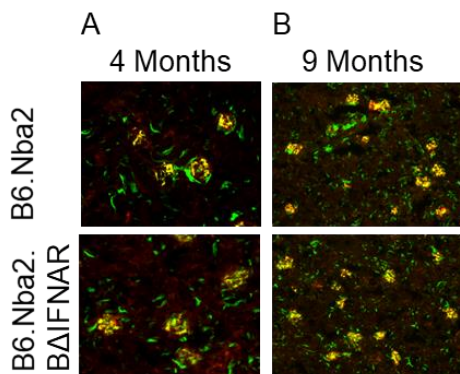

Figure 17: B6.Nba2.BΔIFNAR mice IgG and C'3 deposition is similar to B6.Nba2 at both 4 and 9 months. A-B) Kidneys from B6.Nba2 and B6.Nba2.BΔIFNAR mice were stained with anti-IgG (Red)/anti-C'3 (Green). Two separate cohorts of mice at 4 month of age (A) and 9 months of age (B) were used to compare over time. Each symbol represents the average score per mouse and data are shown as Mean ± SEM. 4 month old mice n = 11 (B6.Nba2); n = 10 (B6.Nba2.BΔIFNAR); 9 month old mice B6.Nba2 (n = 10) and B6.Nba2.BΔIFNAR (n = 8) . * $p < 0.05$, Student's unpaired t-test with Welch's correction.

in the younger mice, points towards a significantly greater pathogenic contribution by IFN-I stimulated B cells in early disease.

Conclusion and Discussion

The B6.Nba2.BΔIFNAR model shows a partial protection from the spontaneous lupus-like disease that develops in this strain. This partial protection allows us to see how IFN-I stimulation of B cells contributes to disease. We find that IFN-I stimulated cells contribute significantly to the production of autoantibodies and have a particularly strong impact towards the production of IgG_{2C} subtype autoantibodies specifically. Unsurprisingly due to their respective roles in antibody production via the germinal center reaction, IFN-I stimulation on B cells also led to increased populations of PCs, Memory B Cells, and GC B Cells. The impact of IFN-I on this process was not largely through alterations of cytokine production, rather through the effect of prolonging cell survival through regulating factors involved in apoptosis. Moreover, IFN-I stimulation on B cells increased levels of activation, via both CD69 and CD40, in B cells. The significant change in splenomegaly also reflected the change in increased populations.

IFN-I stimulation on B cells was not found to contribute to immune complex deposition or complement fixation in the kidneys. We also found that at four months of age IFN-I stimulation of B cells did not impact the size or number of germinal centers, despite the significant effect on the GC B cell population regarding population.

While we had initially hypothesized that IFN-I stimulated B cells were contributing more to disease progression than our data ultimately showed, it is consistent with current published data from other lupus-prone strains. Utilizing a GC B cell-specific IFNAR conditional knock out on the B6.Sle1 spontaneous lupus background, Domier et al

showed that IFN-I stimulation of B cells primarily increased the populations of GC B cells and PC cells [280]. Consistent with our data, they also showed that IFN-I contributed to the production of specific auto-antibodies, in particular those of the IgG2C subtype [280].

In the GC reaction we found that the major contribution of IFN-I stimulation of B cells at 4 months of age was to regulate *Bcl2, Bcl-xl*, and *Bim* in the GC B cells. This finding is consistent with other studies that show IFNα can directly increase the expression of *Bcl2* [276,281]. These transcripts lead to increased survival of the GC B cells [282]. Furthermore, increased GC B cell survival has been found to link to other IFNα-stimulated B cell disease presentations observed in this study.

While initially unexpected, the discovery that IFN-I stimulated B cells contribute primarily to the production of specific IgG2c autoantibodies, via the GC reaction and the Memory B and PC cell subsets produced via this reaction, agrees with data showing that somatic hypermutation may be increased with longer-lived GC B cells [283]. Additionally, IFNα B cell stimulation contributing to production of IgG2c autoantibodies coincides with published literature on both IFN-I and mouse models of lupus-like disease [271,280]. Increased survival of GC B cells, by increasing *Bcl2* expression via transgene, has also been shown to lead to an abundance of memory B cells [284].

Preliminary data from 9 month old B6.Nba2.BΔIFNAR mice and unpublished data from our lab indicate that while IFN-I signaling is required for disease, the pathogenic effect is strongest early in disease. We therefore hypothesize that B6.Nba2 lupus-like disease develops in two phases. The first phase is an IFNα-driven development phase in which

IFNα leads to the upregulation of activation, the germinal center reaction, and the production of auto-antibodies. In this phase of disease, intervention of IFNAR signaling can slow disease progression. The second phase is driven by the immune cell dysfunction created in phase one and can perpetuate disease progression even in the absence of IFNα. This phase uses immune complexes to maintain an inflammatory response and cytokines, such as TNFα or IFNγ, produced by cell populations that were expanded during the IFNα-dependent phase. Intervention of IFNAR signaling in this phase cannot halt disease progression, even temporarily.

Finally, given the partial protection observed, the research from our study and the Domier study point to the impact of IFN-I on another cell subset contributing significantly to disease progression, in addition to the effect on B cells [280]. As T cells are heavily involved in the differentiation of B cells, we speculated that IFN-I stimulation of T cells would be the second IFN-I-driven contribution to pathogenesis in B6.Nba2 mice.

CHAPTER 4: TΔIFNAR

Hypothesis and rationale

The T cell compartment is dysregulated in both human and murine lupus disease. Increased populations of Eff/Mem T cells are observed in patients and in multiple animal models [33,61,85,122,123]. This is accompanied with an increase in activated T cells [33,39,61,85]. The B6.Nba2 model, along with other animal models of lupus, present with not only increased levels of activated B cells as shown above, but also with elevated levels of activated T cells [39,61,85].

Literature has highlighted the increase in TFH cells in SLE patients [124–126]. As TFH cells play an important costimulatory role in the germinal center reaction, they can

contribute to generation of GCs and the differentiation of Memory B cells, PCs, subsequently leading to increased levels of ANAs. Considering the fairly conserved increase in ANAs and germinal center reaction-associated cells in both human and murine lupus, and the relation of IFNα to disease, it is possible that the increase in TFH cells is related to IFNα stimulation. IFNα stimulation of T cells has also been shown to prolong the life of T cells [285], mimicking our observations from B-cell specific IFNAR-deficient mice [33]. Based on these observations, we hypothesized that:

IFNα stimulation of T cells contribute to increased T cell activation, increased Teff/mem populations, and increased levels of TFH cells in B6.Nba2 lupus-like disease.

Results: Disease Phenotype

Thymocyte development is altered in B6.Nba2 lupus-like disease, but is independent of IFN-I T cell stimulation

We assessed thymic development of T cells in B6.Nba2 and B6.Nba2.TΔIFNAR mice by measuring populations of CD4- CD8- double negative (DN), CD4+ CD8+ double positive (DP), CD4+ single positive (SP), and CD8+ SP thymocytes by flow cytometry. B6.Nba2.TΔIFNAR mice did not show significant differences in any of the four major developing thymocyte populations (Figure 18B-E). While determining that IFN-I stimulation on T cells did not significantly contribute to thymocyte development, we also made the novel discovery that thymocyte development is altered as part of the B6.Nba2 lupus-like disease progression. We observed no difference in the number of total thymocytes in B6.Nba2 mice as compared with B6 mice (Figure 18A). When thymocyte populations were compared to that of a B6 wildtype mouse, the B6.Nba2 mice were found to have significantly larger populations of DP thymocytes (Figure 18C).

Oppositely, DN, CD4 SP, and CD8 SP populations showed hints of decreasing in B6.Nba2 versus B6 mice, although these differences did not reach statistical significance

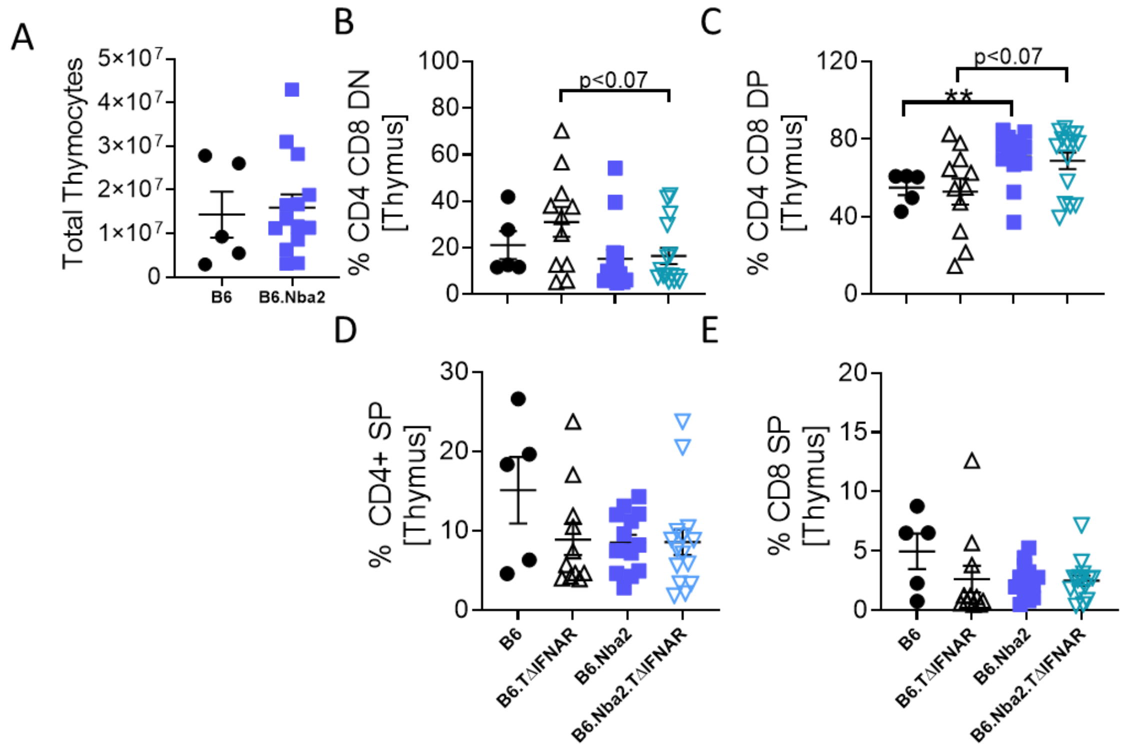

Figure 18: B6.Nba2 mice display smaller thymi, but increased levels of Double Positive Thymocytes. Thymi were measured by splenocyte count (A). Thymocyte development was analyzed using flow cytometry and populations of double negative (DN; CD4- CD8-), double positive (DP; CD4+ CD8+), and single positive (SP; CD4+ or CD8+) were quantified (B-E). B6: n = 5, B6.TΔIFNAR: n = 11, B6.Nba2: n = 13, B6.Nba2.TΔIFNAR: n = 14. Each symbol represents one mouse and data are shown as Mean ± SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction.

(Figure 18B, D-E).

B6.Nba2.TΔIFNAR mice retain classic lupus-like disease presentations of splenomegaly and ANAs

Comparing B6.Nba2 and B6.Nba2.TΔIFNAR mice, there were no significant differences in spleen weight or splenocyte count in response to IFNα stimulation of T cells, although both strains of mice displayed significantly larger spleens than B6 and B6.TΔIFNAR mice (Figure 19A-B). Similarly, B6.Nba2.TΔIFNAR mice were not protected from the development of serum anti-dsDNA autoantibodies, showing similar concentrations of both total IgG and IgG subtypes (1, 2b, 2c, and 3) to their B6.Nba2 counterparts (Figure

19C-D). Non-lupus B6.TΔIFNAR mice maintained similar low levels of anti-dsDNA IgG antibodies as B6 control non-autoimmune mice, allowing us to conclude that IFNα stimulation of T cells is not significantly contributing to splenomegaly or elevated levels of serum anti-dsDNA IgG and IgG subtypes.

Splenic CD4+ T cells subsets and Activated T cells are not altered in TΔIFNAR mice.

Splenocytes were separated into CD4+ and CD8+ T cells using flow cytometry. CD4+ T cells as a whole did not differ, regardless of T cell specific IFNAR expression (Figure 20A). Total splenic CD8+ T cells were reduced in all mice bearing the Nba2 lupus susceptibility locus (Figure 20B). When CD4+ T cell subsets were further analyzed,

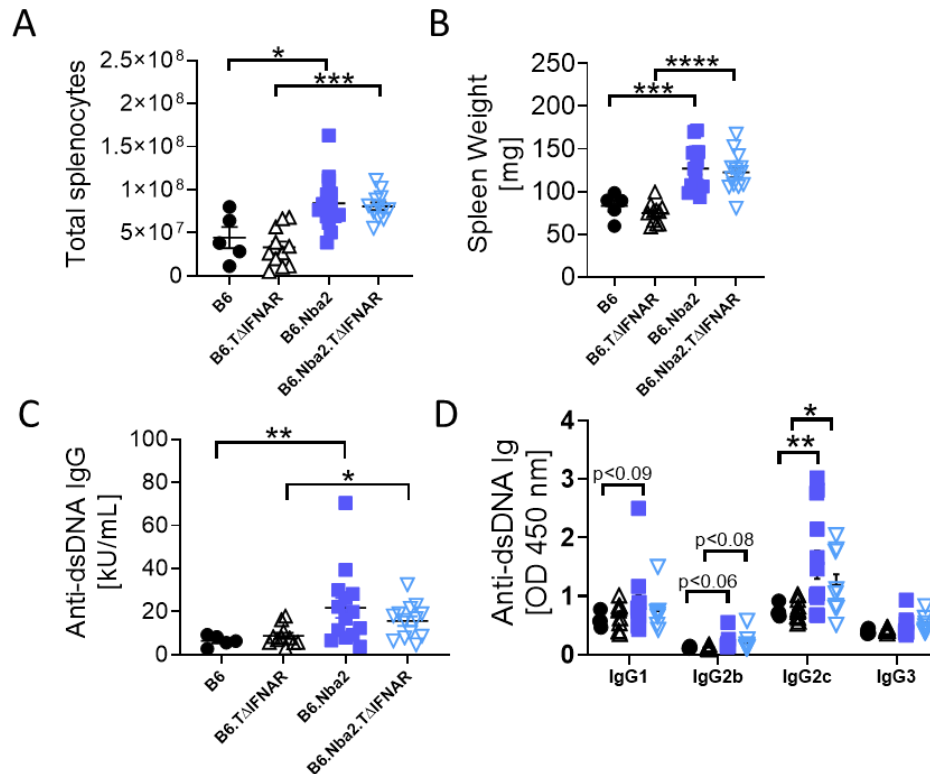

Figure 19: Splenomegaly and autoantibody production is intact in B6.Nba2.TΔIFNAR mice. Splenomegaly was determined by measuring splenocyte count (A) and spleen weight (B) at 4 months of age. Presence of anti-dsDNA IgG and anti-dsDNA IgG subtypes in serum were measured by ELISA (C-D). B6: n = 5, B6. TΔIFNAR: n = 11 B6.Nba2: n = 13, B6.Nba2.TΔIFNAR: n = 14,. Each symbol represents one mouse and data are shown as Mean ± SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction.

naïve and effector/memory populations were found to be similarly skewed towards higher levels of effector/memory CD4+ T cells in B6.Nba2 and B6.Nba2.TΔIFNAR mice (Figure 20C-D). In contrast, non-lupus prone mice retained a higher naïve: eff/mem ratio compared to B6.Nba2 mice (Figure 20C-D). In B6, but not B6.Nba2, mice naïve: eff/mem T cell ratio did differ by T cell-specific IFNAR expression, as B6.TΔIFNAR mice showed a further elevated naïve: eff/mem ratio than B6 mice (Figure 17C-D). This data indicates that while a direct IFNα stimulation of T cells can aid in transition from naïve to the eff/mem type in B6 mice, T cell IFNAR expression is not required to drive this transition in B6.Nba2 mice. Finally, populations of recent and sustained activation markers on splenic T cell were quantified. Recently activated CD69+ CD4+ T cells were found to be elevated in both the B6.Nba2 and B6.Nba2.BΔIFNAR mice, consistent with the *Nba2* locus driven disease phenotype (Figure 20E-F). Intrasplenic levels of IL-2, a main T cell supportive cytokine, were elevated in the B6.Nba2 mice, but this increase was not driven by IFNα (Figure 20G).

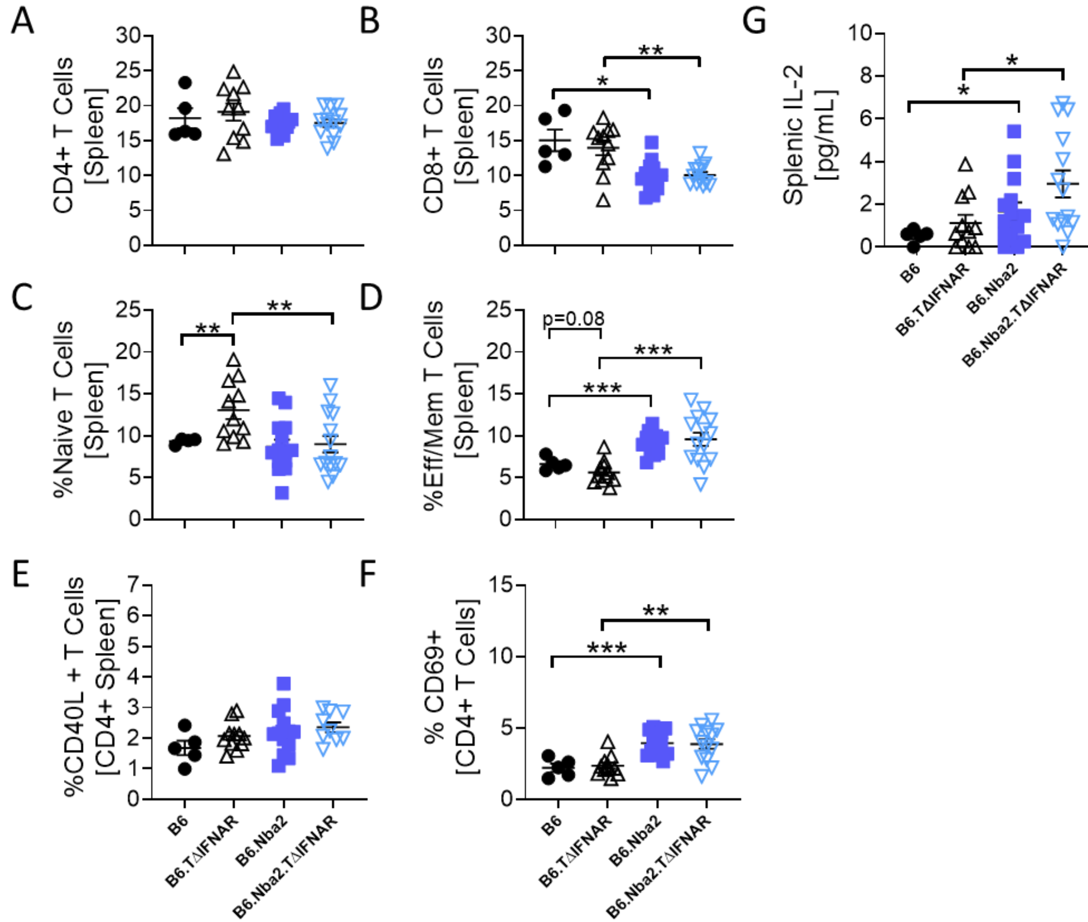

Figure 20: B6.Nba2 mice display increased levels of Effector/Memory T cells and CD69+ Activated T cells, but decreased levels of total CD8+ T cells. Splenic cell populations in 4 month old B6, B6. TΔIFNAR, B6.Nba2, and B6.Nba2.TΔIFNAR mice were quantified using flow cytometry. Populations of single positive T cells were gated as CD4+ or CD8+ (A-B). CD4+ T cells were further classified as Naïve CD4+ T cells (CD62L^{high}CD44^{low}), Effector/Memory CD4+ T cells (CD44^{high}CD62L^{low}), CD40L+ CD4+ T cells (CD40L+CD4+), and Activated CD4+ T cells (CD69+CD4+) (C-F). Levels of splenic IL-2 were quantified in spleen supernatant using U-Plex Cytokine Array (G). B6: n = 5, B6. TΔIFNAR: n = 11 B6.Nba2: n = 13, B6.Nba2.TΔIFNAR: n = 14,. Each symbol represents one mouse and data are shown as Mean ± SEM. * p < 0.05; ** p < 0.01; **** p < 0.0001; Student's unpaired t-test with Welch's correction.

T cell skewing in TΔIFNAR mice is independent of IFN-I stimulation.

When specific CD4+ T cell subsets were further examined, both B6.Nba2 and B6.Nba2.TΔIFNAR mice were found to express increased levels of Th1 and Th17 cells as compared to B6 and B6. TΔIFNAR mice (Figure 21A-B). The cytokine milieu in the spleen was further measured utilizing an electrochemiluminescence array on spleen supernatant. Interestingly, B6.Nba2 and B6.Nba2.TΔIFNAR mice displayed the same significant increased levels of IFNγ as compared with non-autoimmune mice, while no change was observed in levels of IL-17A. Thus, the increased populations of TH1 and TH17 cells and elevated levels IFNγ observed in lupus-like disease are not a direct result of IFN-I stimulation on the T cells (Figure 21C-D).

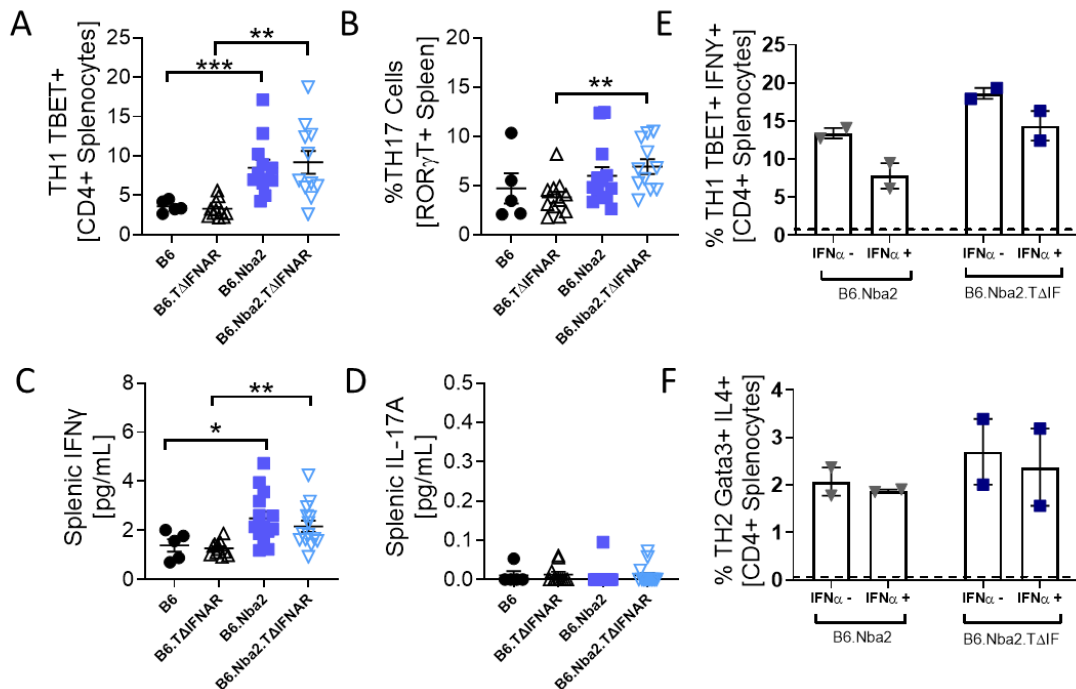

Figure 21: B6.Nba2 mice have increased Th1 cells and Th17 cells and increased splenic IFNγ. CD4+ T helper subsets TH1 and TH17 were measured by flow cytometry using intracellular staining for master transcription factors, Tbet and RorγT, respectively (A-B). Splenic cytokine levels of IFNγ and IL-17A were measured in spleen supernatant by U-Plex mouse biomarker assay (C-D). The ability of naïve T cells to skew to a helper phenotype was assessed by incubating naïve T cells with cytokine cocktails designed to skew T cells *ex vivo* in the presence or absence of IFNαA. TH1 and TH2 populations were measured post incubation by intracellular flow cytometry for master transcription factor and cytokine production (E-F). Stippled line represents unstimulated cells (E-F). B6: n = 5, B6. TΔIFNAR: n = 11 B6.Nba2: n = 13, B6.Nba2.TΔIFNAR: n = 14. Each symbol represents one mouse and data are shown as Mean ± SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction.

Naïve CD4+ T cell cytokine-dependent transition to Th1 and Th2 is not affected by IFN-I

To further verify a lack of IFNα-driven skewing of CD4+ T cell subsets, *ex vivo* skewing of naïve T cells was performed in the presence or absence of recombinant IFNαA. Naïve CD4+ T cells were isolated from spleens of B6.Nba2 and B6.Nba2.TΔIFNAR mice and co-cultured with Th1 or Th2 skewing cytokines in the presence or absence of recombinant IFNαA. No difference was found in the population of transitioned cells, as measured by intracellular flow cytometry for master transcription factors Tbet and Gata3, IFNγ and IL-4 (Figure 21E-F). Naïve CD4+ T cells from both strains of mice cultured in the absence of Th1 and Th2 skewing cytokines did not differentiate to TH1 and TH2 cells (shown as stippled line). Therefore, direct IFNα stimulation of T cells does not contribute to the overall increased TH1 and TH17 skewing observed in B6.Nba2 and B6.Nba2.TΔIFNAR mice.

Increased TFH cells in B6.Nba2 mice are not a direct result of IFNα stimulation

Consistent with previously published data, B6.Nba2 mice had elevated levels of splenic TFH cells, identified as CD4+ PD1+ CXCR5+, which were independent of IFNAR expression on T cells (Figure 22A-B). In contrast, Foxp3+ Tcregs were found to be similarly expressed in B6.Nba2 and B6.Nba2.TΔIFNAR mice (Figure 22C). Finally, cytokines produced by TFH cells including IL-21, IL-4, and IL-10, the latter also produced by CD4+ Tregs, were elevated in spleen supernatants from both B6.Nba2 and B6.Nba2.TΔIFNAR mice (Figure 22D-F), suggesting that the increased splenic TFH population and elevation of associated cytokines found in the B6.Nba2 lupus-like disease model is not a direct result of IFNα stimulation of T cells.

IFN-I directly contributes to limiting the CD8 Tcreg population in lupus like disease

As the CD4+ T cells had provided no indication of a pathogenic disease contribution in direct response to IFN-I, we sought to determine if the same was true for CD8+ T cells.

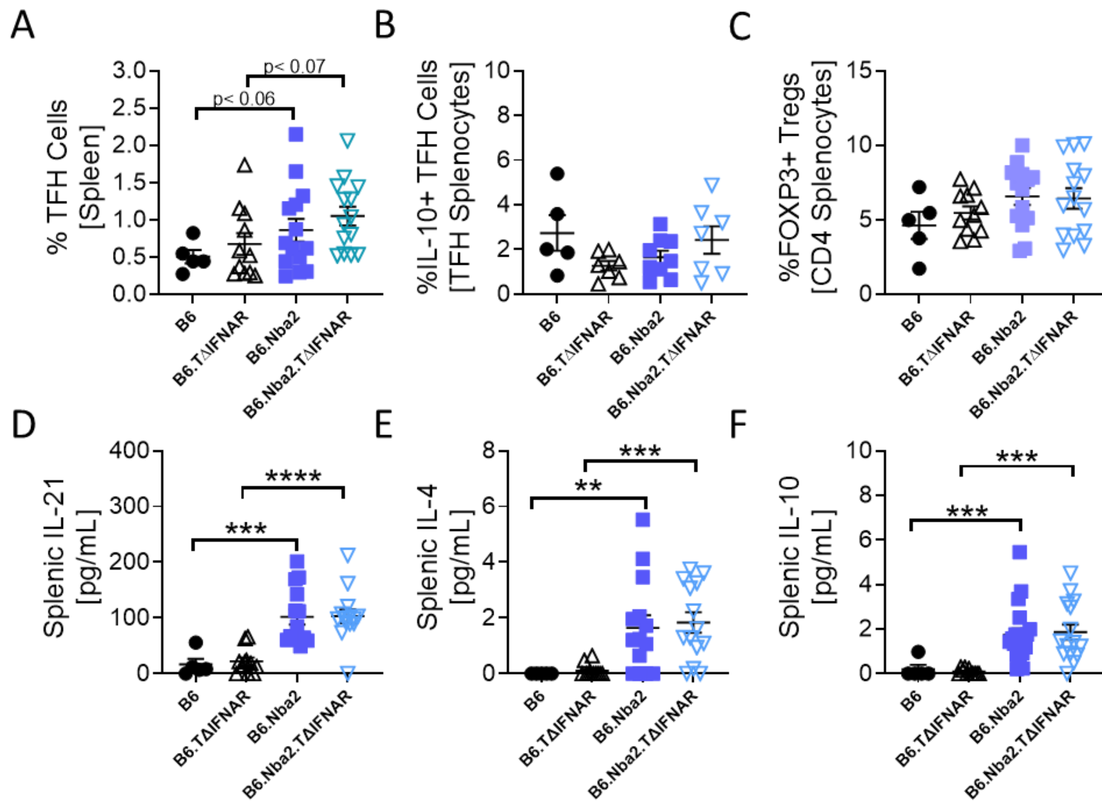

Figure 22: Increased levels of TFH cells and IL-21 in B6.Nba2 mice are independent of IFNAR expression. TFH cells were measured using flow cytometry as CD4+ PD1+ CXCR5+ (A). IL-10 producing TFH cells were further identified as IL-10+ by intracellular staining (B). Treg populations were identified by expression of FoxP3+ (C). Splenic cytokine levels of IL-21, IL-4, and IL-10 were measured by U-Plex mouse biomarker assay (D-F). B6: n = 5, B6. TΔIFNAR: n = 11, B6.Nba2: n = 13, B6.Nba2.TΔIFNAR: n = 14. Each symbol represents one mouse and data are shown as Mean ± SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction.

While less frequently discussed, CD8+ T cells do in fact present as different subtypes of cytotoxic T cells (Tc) [120,121]. Similar to their CD4+ counterparts, they upregulate master transcription factors and produce cytokines, in addition to their cytotoxic abilities [120,121]. In fact, they can upregulate the same transcription factors as CD4+ T cells [120]. Due to the decreased splenic CD8+ T cell population observed in B6.Nba2 and B6.Nba2.TΔIFNAR mice (Figure 23A), we further analyzed the CD8+ cytotoxic T cell

subsets. Both B6.Nba2 and B6.Nba2.TΔIFNAR mice displayed significantly increased populations of Tc1 (CD8+ Tbet+) cells as compared with non-autoimmune B6 and B6.TΔIFNAR mice (Figure 23B). In contrast, Tc17 (CD8+ RORγT+) cells remained unaltered regardless of background or T cell ability to respond to IFN-I (Figure 23C). Surprisingly, B6.Nba2.TΔIFNAR mice displayed larger populations of Tcreg (CD8+ FOXP3+) splenic T cells when compared to both B6.TΔIFNAR and B6.Nba2 mice (Figure 23D), indicating that, IFN-I stimulation of T cells could directly contribute to a reduction in the population of splenic CD8+ FOXP3+ cells.

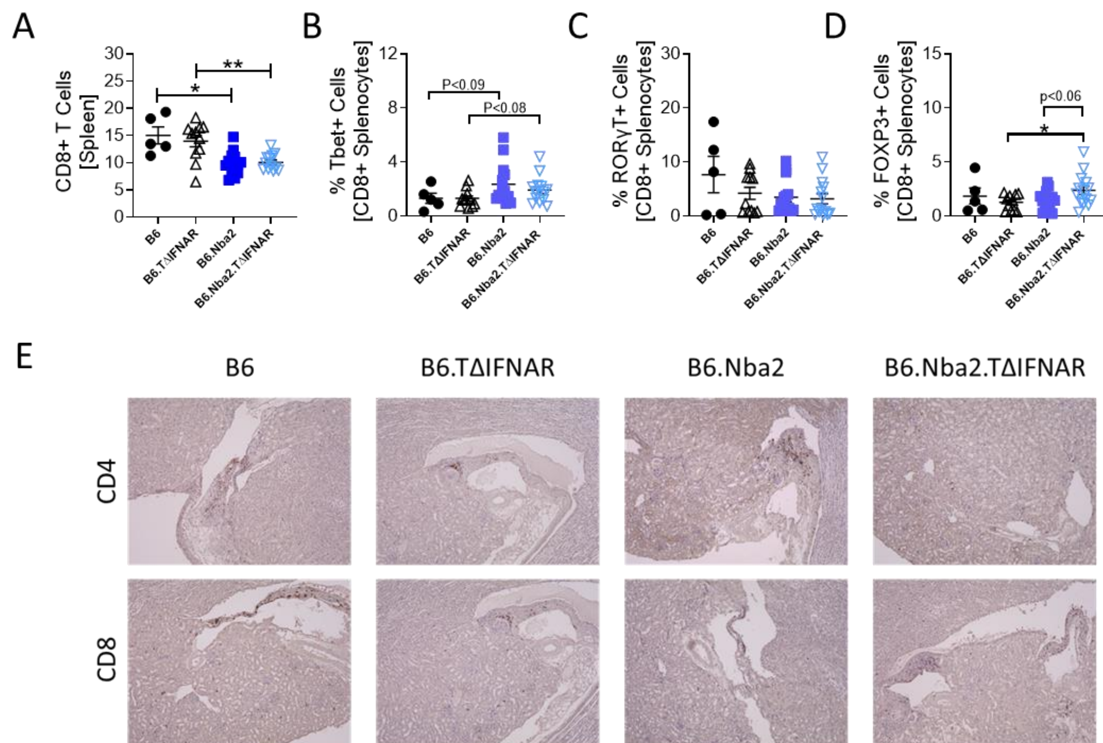

Figure 23: B6.Nba2 mice display reduced levels of total CD8+ T cells, but increased frequencies of Tc1 cells. Populations of CD8+ cells were quantified using flow cytometry (A). Cytotoxic CD8+ cells were further analyzed by intracellular staining for master transcription factors Tbet (B), Rorγt (C), Foxp3 (D). Numbers, age, statistics. B6: n = 5, B6. TΔIFNAR: n = 11 B6.Nba2: n = 13, B6.Nba2.TΔIFNAR: n = 14. Paraffin embedded kidneys were stained with CD4 and CD8; seen in brown (E). B6: n = 2, B6. TΔIFNAR: n = 2 B6.Nba2: n = 4, B6.Nba2.TΔIFNAR: n = 5. Each symbol represents one mouse and data are shown as Mean ± SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction.

Literature shows that often times the T cells that infiltrate the organs, causing inflammation and damage, in lupus-like disease are CD8+ cytotoxic T cells [286–288]. A reduction in splenic CD8 T cells could be a result of increased migration to the organs, in particular the kidneys. To determine if the decrease in splenic CD8+ T cells was a result of increased CD8+ T cell infiltration in the kidneys we stained fixed kidney tissue with antibodies for CD4 and CD8. Preliminary staining for CD4+ and CD8+ T cells (brown) in the kidneys did not show any obvious differences between the two strains (Figure 23E).

Conclusion and Discussion

We have previously shown that IFN-I stimulation of B cells results in reduced B cell activation, GC formation, plasma cell differentiation and autoantibody production in B6.Nba2 mice. We speculated that IFN-I stimulation of T cells would similarly drive aspects of lupus-like disease. We show here that surprisingly, IFN-I stimulation of T cells contribute minimally to disease pathogenesis in this model. As B6.Nba2 lupus-like disease presents with a dysregulated T cell compartment, we had incorrectly hypothesized that IFN-I stimulated T cells were directly contributing to some, if not all, of that dysregulation. Instead, we found that the T cell compartment remained largely the same, regardless of the T cell's ability to respond to IFN-I signaling.

Interestingly, the only difference we observed was elevated levels of splenic Tcreg (CD8+ FoxP3+) cells in B6.Nba2.TΔIFNAR mice, suggesting that IFN-I may be able to directly suppress or reduce the population of splenic CD8 Tregs. CD8+ FOXP3+ cells, similar to CD4+ FOXP3+ cells, are also regulatory cells. CD8 Tregs have been reported to express increased CTLA-4, ICOS, and Ki67 and suppress proliferation of effector T

cells [289]. It remains to be determined if this effect is mediated via regulation of apoptosis markers as previously seen in B cells, or due to altered proliferation. However, literature shows that IFNα can act as the "third" cytokine, in addition to IL-12 and IFNγ, in the differentiation of CD8+ effector cells [290–296].

Decreased longevity of IFNAR-expressing Foxp3+ CD8+ Tcregs could subsequently allow for increased proliferation of T effector cells during disease development. As ANAs are a strong marker of disease in the B6.Nba2 lupus-prone model, the conclusion can be drawn that while TΔIFNAR mice are able to generate a larger population of splenic CD8+ Tcregs than WT mice, this population is not enough to break the cycle of autoimmunity on its own.

In conclusion, the skewing of the CD4+ T cell compartment in B6.Nba2 lupus prone mice is not a direct consequence of IFN-I stimulation on T cells. However, this study highlights a previously understudied role for CD8 T cells, particularly Tcregs, SLE. If further research shows that Tcregs can curtail an autoimmune response, they may play an integral role in reducing established disease.

CHAPTER 5: MyΔIFNAR

Hypothesis and rationale

As discussed in previous sections, ANA presence in SLE patients is one of the most ubiquitous symptoms in an extremely heterogeneous disease. However, low levels of ANAs can be found in healthy patients too, as basal levels of cell death are not unique. Therefore, researchers have studied how ANAs develop in SLE patients and the distinction from the how low levels appear in healthy patients. As such, correlations

between serum anti-dsDNA autoantibodies, elevated IFN-I levels and renal disease has been well-established [1,184,197–200].

In addition to elevated ISG expression, SLE patients have also been shown to have a granulopoiesis-associated signature [1]. Later research showed that netosis, a method of neutrophil death described in the introduction, was able to induce the production of IFN-I in SLE patients [297]. Monocyte and netosis-induced IFN-I production has been observed both in the pristane lupus mouse model [174] and in the non-lupus immune disease Wiskott Aldrich Syndrome [298]. Neutrophils ability to facilitate induction of IFN-I by netosis combined with research implications that increased NET production may be source of nuclear material to drive ANA production in SLE patients has led researchers to hypothesize that myeloid cells are pathogenically linked with IFN-I. Interestingly, research has showed that NETs are linked with other SLE disease presentations, such as vasculitis and tissue damage, particularly in the kidneys [169–173]. Finally, myeloid cells are instrumental in the removal of apoptotic cells, another potential source of nuclear antigen in SLE, both in tissues and in secondary lymphoid organs [183]. Thus, we hypothesize that:

Pathogenic disease presentations of netosis, vasculitis, kidney damage, and ANA production are a direct consequence of IFNα stimulation of myeloid cells.

To address this hypothesis, we developed IFNAR$^{flx/flx}$ LysM-cre/cre B6.Nba2 mice and studied lupus-like disease development in 4 month old animals.

Results: Disease Phenotype

IFN-I signaling in LysM+ myeloid cells affect myelopoiesis in the bone marrow.

In order to assess lupus like disease development in B6.Nba2.MyΔIFNAR mice we sought to determine if the immune cell development process differed due to the inability of lysosome M expressing cells to respond to IFN-I. There was no difference in the total bone marrow cell number between B6.Nba2 and B6.Nba2.MyΔIFNAR mice (Figure 24A). Further analysis of bone marrow stem cell and progenitor cell populations (hematopoietic stem cells (HSCs), common myeloid progenitors (CMPs) granulocyte/macrophage precursors (GMPs), megakaryocyte/erythrocyte precursors (MEPs), and common lymphoid progenitors (CLPs)) were quantified by flow cytometry using the gating strategy shown in Figure 24B. B6.Nba2.MyΔIFNAR mice showed a trend towards increased CLP cells, indicating that bone marrow development may be shifted towards common lymphoid progenitors when LysM+ myeloid cells cannot respond to IFN-I (Figure 24C-F). Interestingly, we saw significantly increased populations of immature B cells, but no differences among pre-pro B or pro B cells, in the bone marrow of MyΔIFNAR mice (Figure 24G-I).

We further examined mature cells in the bone marrow and found no significant difference in total CD11b+ positive cells (Figure 24J), but a significant decrease in monocytes and a trend towards reduced levels of bone marrow neutrophils (Figure 24K-L). Interestingly,

there was also a trend towards decreased CD3+ cells in the bone marrow (Figure 24M).

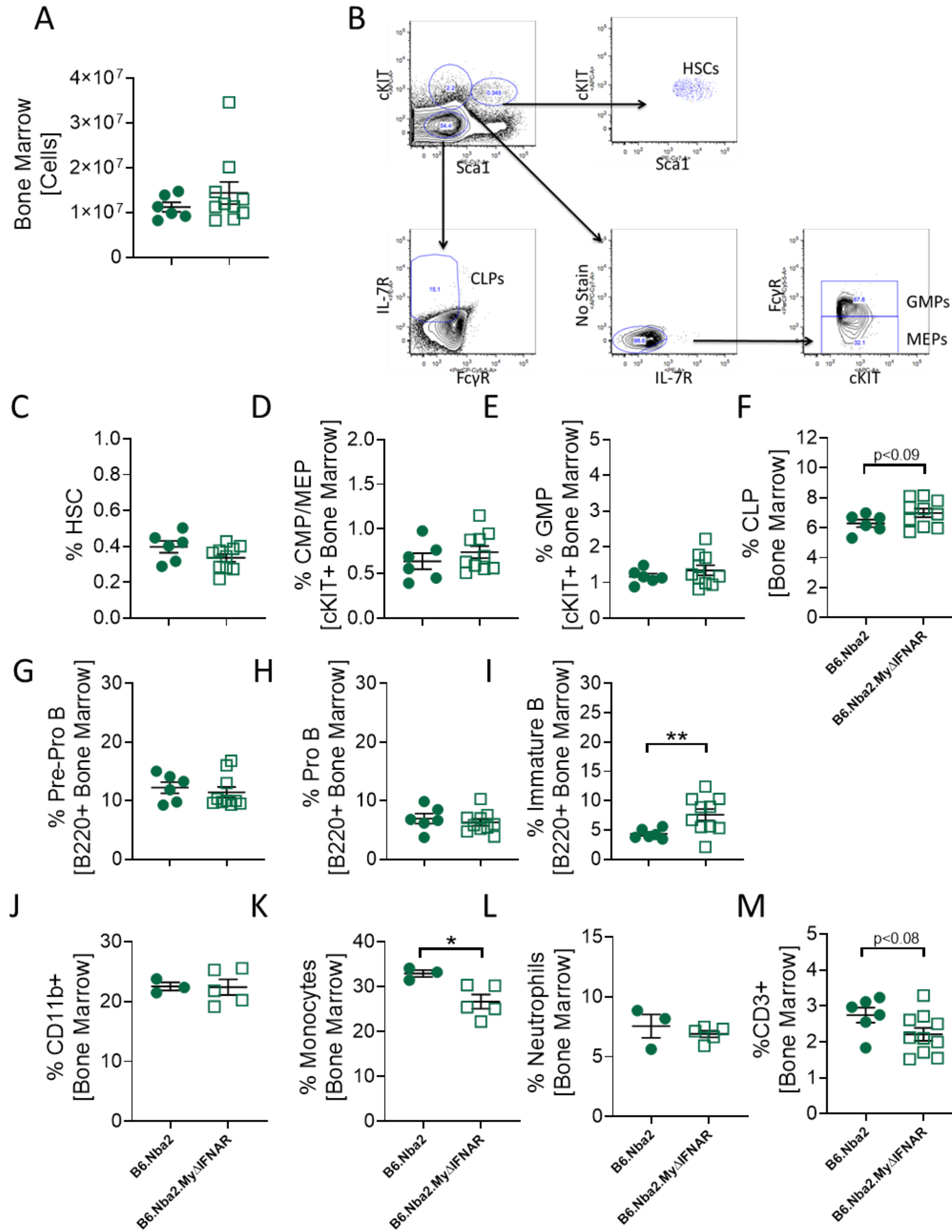

Figure 24: MyΔIFNAR mice have decreased bone marrow monocytes and elevated immature B cells. Bone marrow cell populations were quantified from cells flushed from the left femur and tibia of 4 months old B6.Nba2 (n = 6) or B6.Nba2.MyΔIFNAR (n = 10) mice (A). Early progenitor populations were isolated according to the gating strategy in (B). Myeloid progenitors were quantified using flow cytometry and separated into four populations; hematopoietic stem cells (HSCs), common myeloid progenitors/megakaryocyte erythrocyte progenitors (CMPs/MEPs), granulocytic myeloid progenitors (GMPs), and common lymphocyte progenitors (CLPs) (C-F). Pre-pro B cells (CD43+ IgM- CD19-) (G), Pro B cells (CD43+ CD19+ IgM-) (H) and immature B cells (CD43- IgM+ AA4.1+) (I) were determined. A subset of mice were also tested for levels of CD11b+ cells, Ly6C+CD11b+ monocytes, Ly6G+CD11b+ neutrophils and CD3+ T cells (J-M): B6.Nba2 (n = 3) and B6.Nba2.MyΔIFNAR (n = 5). Each symbol represents one mouse and data are shown as Mean ± SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction.

Whether these cells represent T cell precursors or re-circulating T cells remains unknown.

MyΔIFNAR mice have significantly increased recently activated T cells

We further determined disease progression in these mice by quantifying splenomegaly and splenic cell populations. B6.Nba2.MyΔIFNAR mice were not protected from splenomegaly when measured by both weight and splenocyte count (Figure 25A-B). There were no differences in total splenic B cells, CD4+ T cells, or CD8+ T cells despite the increase in immature B cells and decrease in CD3+ T cells found in the bone marrow

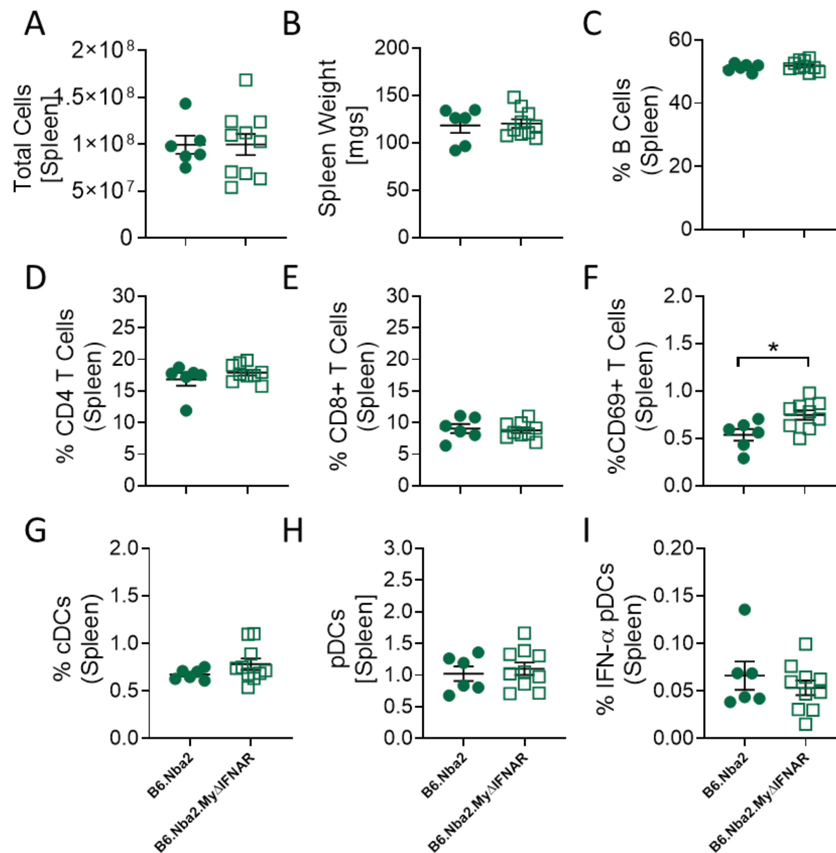

Figure 25: MyΔIFNAR mice do not have skewed splenic B cells, T cell, or DCs, but have significantly increased CD69+ T cells. Splenomegaly was quantified by spleen count (A) and spleen weight in 4 months old B6.Nba2 (n = 6) or B6.Nba2.MyΔIFNAR (n = 10) mice. Splenocyte subsets were quantified using flow cytometry. B cells were gated using surface marker B220 (C), and T cells were gated using markers CD4 and CD8 (D,E). Recently activated CD4+ cells were gated as CD69+. Dendritic cells (CD11c+) were separated into CD11b+ (pDCs) and CD11b- (cDCs) (G,H). IFNα pDCs were classified as SigH+ PDCA1+ (I). Each symbol represents one mouse and data are shown as Mean ± SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction.

(Figure 25C-E). In contrast, B6.Nba2.MyΔIFNAR had significantly increased populations of CD69+ recently activated splenic CD4+ T cells (Figure 25F). This increase was of particular interest as elevated CD69+ activated T cells have previously been observed in B6.Nba2 mice [21,33,39,61,299]. Populations of CD11c+ splenic dendritic cells, both CD11b- (cDCs) and CD11b+ (pDCs) were found to be unchanged (Figure 25G-H). Consistent with the finding that splenic pDC populations were unaltered, we found IFNα-producing pDCs, identified as SigH+ PDCA1+, to be similar between B6.Nba2 and B6.Nba2.MyΔIFNAR mice (Figure 25I).

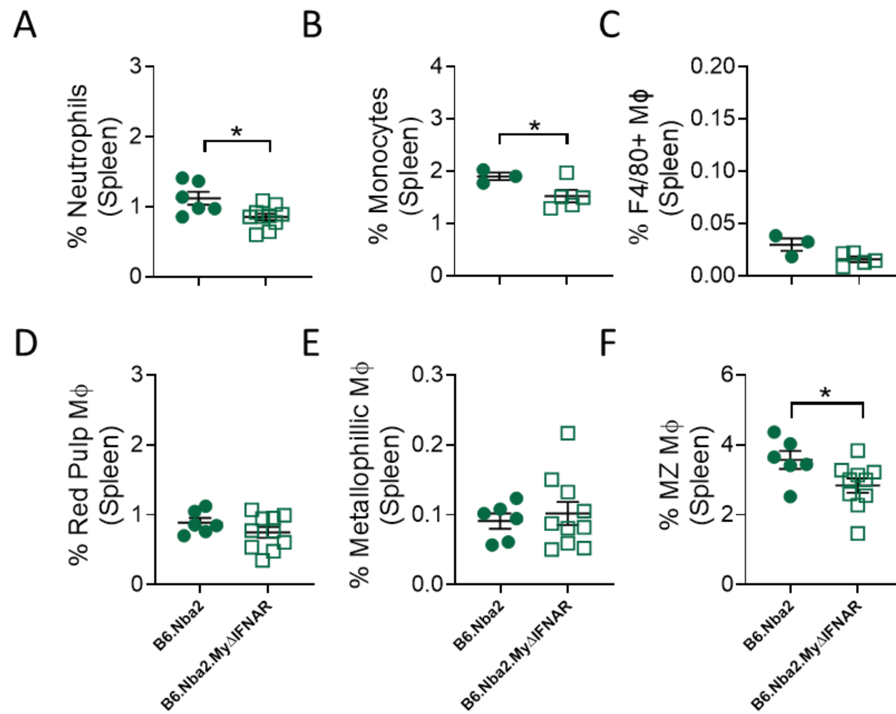

Figure 26: MyΔIFNAR mice have reduced populations of splenic neutrophils, monocytes, and marginal zone macrophages. Splenic populations of neutrophils (Ly6G+CD11b+) and monocytes (Ly6C+CD11b+) were quantified using flow cytometry (A-B) from B6.Nba2 (n = 3-6) and B6.Nba2.MyDIFNAR (n = 6-10). Splenic macrophages were divided into subpopulations determining F4/80+ macrophages(C), red pulp macrophages (F4/80 hi CD11b int)(D), metallophilic macrophages (MOMA+)(E) and MZ macrophages (SignR1/MARCO+) (F). Each symbol represents one mouse and data are shown as Mean ± SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction.

MyΔIFNAR mice had reduced populations of splenic myeloid cells

Consistent with the reduced levels of monocytes in the BM, we found that splenic myeloid cell populations were significantly altered in MyΔIFNAR mice. As such, populations of splenic neutrophils and monocytes were significantly decreased in MyΔIFNAR mice (Figure 26A-B). We found no significant difference in the small population of splenic F4/80+ macrophages (Figure 26C). Specialized macrophages play different immune roles so we further quantified populations of red pulp macrophages, metallophilic macrophages, and MZ macrophages. No difference was seen in populations of red pulp macrophages or metallophilic, MOMA+ macrophages (Figure 26D-E). However, there was a significantly decreased population of SignR1+ MZ macrophages

(Figure 26F), suggesting that IFN-I signaling is involved in the development or maintenance of this population of macrophages.

MyΔIFNAR mice have elevated populations of GC-reaction associated B cells

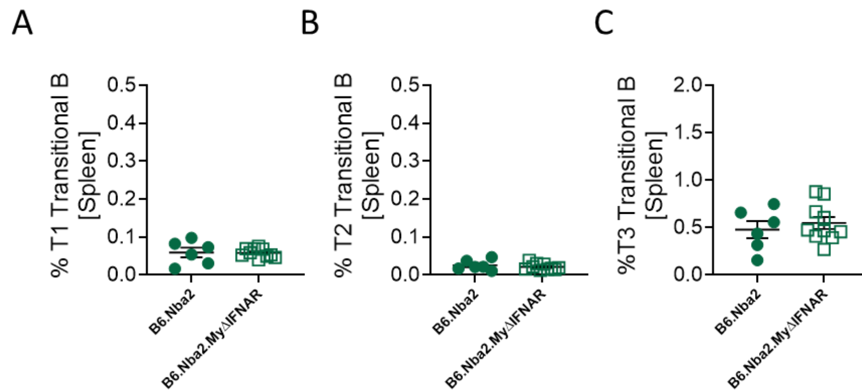

Figure 27: MyΔIFNAR mice do not differ in transitional B cell populations. Transitional B cell populations in the spleen were quantified using flow cytometry. All transitional cell subsets were identified as B220+ AA4.1+ and further divided into T1 (IgM- CD23+), T2 (IgM+ CD23+), and T3 (IgM+ CD23-) subsets (A-C). B6.Nba2 (n = 6) or B6.Nba2.MyΔIFNAR (n = 10) Each symbol represents one mouse and data are shown as Mean ± SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction.

MyΔIFNAR mice showed an increase in immature B cells in the bone marrow, prompting us to investigate splenic B cell populations. Surprisingly we found no significant differences in subsets of transitional B cells (Figure 27A-C). We observed a reduction in the population of follicular B cells, but no difference in MZ B cells or activated B cells (Figure 28A-D). This data suggests that populations of follicular B cells can be increased as a consequence of IFNα stimulation of LysM+ myeloid cells.

Surprisingly, B6.Nba2.MyΔIFNAR mice showed increased populations of GC reaction-associated B cells; GC B cells, Memory B cells, and PCs (Figure 28E-G), indicating an unexpected role for LysM+ myeloid cells to reduce cell GC-associated B cell populations in response to IFN-I stimulation. The increased population of splenic plasma cells was not mirrored in the long-lived, bone marrow PC population (Figure 28H), which could

indicate that IFNα stimulated myeloid cells do not play a significant role in regulation of long-lived PC populations.

The increase in GC B cells, Memory B cells, and PC cells seen in the B6.Nba2.MyΔIFNAR further indicate that the mice may experience increased

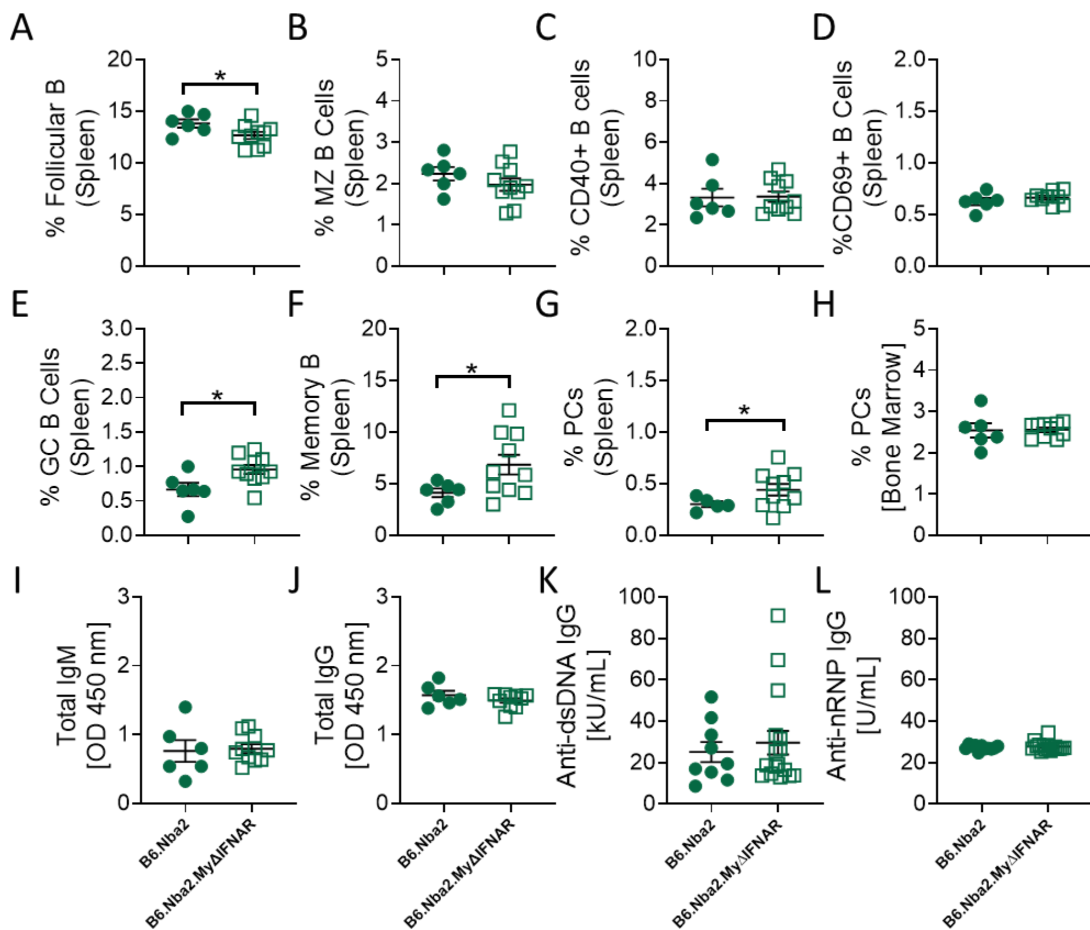

Figure 28: MyΔIFNAR mice display increased germinal center-associated B cell populations. The B cell compartment of the spleen of 4 months old B6.Nba2 (n = 6) or B6.Nba2.MyΔIFNAR (n = 10) mice was determined using flow cytometry (A-G). Plasma cells (PCs) in both the spleen and the bone marrow (long-lived) were identified using CD138 (G-H). Total levels of serum IgG and IgM were quantified using ELISA (I,J). Serum ANA concentration was measured by ELISA (K-L). Each symbol represents one mouse and data are shown as Mean ± SEM. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; Student's unpaired t-test with Welch's correction.

autoantibody levels as well. Surprisingly, the increase in GC-associated B cell

populations was not accompanied by an increase in total IgM or IgG immunoglobulins (Figure 28I-J). As we have previously shown that IFNα specifically plays a role in serum autoantibodies[33], we quantified serum ANAs (anti-dsDNA and anti-nRNP IgG) in B6.Nba2.MyΔIFNAR mice but found no significant difference (Figure 28K-L).

Conclusion and Discussion

Somewhat surprisingly, B6.Nba2.MyΔIFNAR mice showed alterations in splenic populations involved in typical disease progression in B6.Nba2 mice. Populations of splenic neutrophils, monocytes, and MZ macrophages were decreased in B6.Nba2.MyΔIFNAR mice, yet we found significant increases in activated T cells, GC B cells, Memory B cells, and PCs in the spleen. This data points towards a suppressive role of LysM+ myeloid cells as a direct response to IFNα stimulation.

Two possible scenarios fit in with the changes we see in the splenic myeloid populations of B6.Nb2.MyΔIFNAR mice. First, we observed a decrease in SignR1+ MZ macrophages. As MZ macrophages work to remove apoptotic cells from the perifollicular zone [183,300], the decrease in MZ macrophages may have led to an accumulation of dead cells in the MZ. This accumulation of apoptotic cells could then serve as a source of antigen. In experimental autoimmune encephalitis (EAE) apoptotic blebs have been reported to act as an antigen via stimulating TLR9 on B cells, resulting in increased production of IL-10 [300]. Depletion of the SignR1+ MZ macrophages in B6.Act-mOVA-II, a lupus prone model, showed that the MZ macrophages have a critical role in maintaining tolerance to apoptotic cells, and without the MZ macrophages disease was accelerated in the mice [301]. Finally, our observation is consistent with data from another lupus model, where the depletion of the MZ macrophage population increased

disease [301], and with patient data showing an increase in apoptotic cells in the germinal centers that correlated with reduced clearance ability of tingible body macrophages [302]. Future staining of GCs by immunofluorescence or immunohistochemistry, alongside staining for apoptotic cells would help discern this possibility.

Second, we observed a decrease in splenic neutrophils and monocytes, yet no accompanying decrease, rather an increase, in splenic populations associated with lupus-like disease. These observations lead us to believe that the population of myeloid cells that were reduced contained immunosuppressive cells. Our lab has previously shown that a subset of myeloid cells, MDSCs, can suppress lymphocyte activation [175,176] Furthermore, depletion of MDSCs was found to promote autoimmunity in BW mice [175]. Interestingly, MDSCs were found by us and others to reside in the peri-follicular zone, occasionally invading the MZ to interact with MZ macrophages [175,303], although the importance of such interaction remains to be determined. As will be discussed below, further studies evaluating apoptotic cell clearance and the specificity of autoantibodies are required to understand the mechanisms of IFN-I signaling in myeloid cells. In conclusion, the B6.Nba2.MyΔIFNAR mice disease phenotype indicates that the direct result of IFNα stimulation on myeloid cells contributes to increasing populations of MDSCs and MZ macrophages in an attempt to prevent, not drive, autoimmunity.

Interestingly, the increase in GC-associated B cell populations did not correlate with significantly increased serum immunoglobulins, anti-dsDNA IgG, or anti-nRNP IgG. If the defect leading to autoantibody production in B6.Nba2 mice is in fact due to diminished apoptotic cell clearance, it is however possible that autoantibodies with different specificities are affected. Thus, in order to quantify if immunosuppression

provided by the IFNα-dependent MZ macrophages and/or MDSCs affects the full autoantibody repertoire, determination of antibodies specific for other self-antigens needs to be measured in both control B6.Nba2 and B6.Nba2. MyΔIFNAR mice.

In conclusion, the immunosuppressive role shown here by the MZ macrophages and MDSCs presents additional pathways to target in SLE. The MyΔIFNAR model shows that certain IFNα-dependent responses are anti-inflammatory, such as the increase in marginal zone macrophages, but are unable to surmount the strength of the pro-inflammatory response in the diseased state. If we can enhance the tolerant potion of immune response, perhaps we can interfere enough to shut down the autoimmune cycle.

CHAPTER 6: General discussion and Conclusion

Impact of Type I IFN stimulation on B6.Nba2 lupus like disease

While the immune cell subsets studied in this research did not necessarily contribute to disease in direct response to IFN-I as hypothesized, they did provide important insight into how IFNα is contributing to disease pathogenesis- and how it is not. Previous research revealed that SLE patients have increased ISG expression [1], and that IFNα is necessary to drive disease in several of the spontaneous lupus mouse models [19,23,28]. What was previously unknown is how IFNα was working to drive lupus pathogenesis. Adding to the complexity is that many different cells can respond to IFNα, and that the secondary results of IFNα stimulation, particularly by immune cells, can induce a variety of other immune modulators such as cytokine production, gene transcription, cell development, and surface marker expression.

In order to parse out the direct impact of specific immune cells subsets as it relates to driving lupus-like disease pathogenesis, we created a variety of conditional IFNAR

knockouts targeting separate immune cells in a spontaneous lupus-model. Using previously published literature on the effects of IFNα on immune cells in other models, we hypothesized how disease progression in these cell-specific IFNAR knockouts might present. However, our models revealed that the role IFNα plays in lupus pathogenesis is rather complicated.

The B6.Nba2.BΔIFNAR mice showed that IFNα stimulation of B cells directly contributes to the development of splenomegaly [33]. This model also showed that another direct pathogenic effect of IFNα stimulation of B cells is amplification of the GC reaction via the B cells [33]. We showed that in unaltered lupus-like disease IFNα stimulation of B cells directly results in increased populations of activated B cells, GC B cells, Memory B cells, and PCs [33]. Furthermore, the increase of ANAs, particularly the IgG_{2C} subtype, was also shown to be a direct effect of B cell IFNα stimulation [33]. IFNAR-expressing GC B cells show elevated transcription of survival factors, *Bcl2* and *Bclxl*, and down-regulation of pro-apoptotic factor *Bim* as compared to IFNAR-deficient GC B cells [33]. Therefore we were able to conclude that IFNα stimulation of B cells contributes to lupus disease pathogenesis by increasing B cell activation and prolonging the life of GC B cells, allowing for the accumulation of GC B cells, and an increased in post-GC reaction populations of memory B cells and PCs. Furthermore, IFNAR expression on GC B cells specifically allows for class switch recombination (CSR) to the Cγ2c (Cγ2a variant) locus [283], and the combination of increased PCs numbers and CSR to Cγ2c would result in increased total and IgG_{2C} ANAs. Once the increase of PCs was initiated and auto-reactive antibodies were in circulation, the disease may perpetuate via an IFNα-independent inflammatory response to immune complexes.

The B6.Nba2.TΔIFNAR mice gave us some particular insights to the direct pathogenic disease contribution of IFNα stimulated T cells. Surprisingly, we found that many of the pathogenic contributions by T cells to lupus like disease were not a direct effect of IFNα stimulation. While T cells certainly contribute to lupus-like disease progression, the only direct pathogenic effect of IFNα stimulation observed in the B6.Nba2 lupus model was a decrease in the population of CD8+ Tregs. Researchers often consider the CD4+ Tregs and their role of curtailing autoimmunity, however CD8+ Tregs can also contribute to preventing auto-reactivity.

Given our understanding of the effect of IFNα on GC B cell survival via regulation of Bcl2/Bim, it is possible that a similar mechanism would affect T cells. In fact, it was previously published that IFNα is capable of keeping T cells alive after activation [304]. The effect however appears to be different when it comes to CD8+ Tregs, as the population decreases in response to IFNα. Further studies are needed to understand if the effect is direct or if IFNα affects the differentiation process of naïve CD8+ T cells, skewing these away from the regulatory subset. Multiple studies have shown that IFNα can act as a third signal, in addition to IL-12 and IFNγ, to influence the CD8 T cell effector subtype [290–292,294,296,305], so we predict that IFNα is also acting in this manner in our model.

Interestingly, there was a significant difference in the ratio of naïve to effector memory CD4+ T cells, but only on the B6 background, suggesting that IFN-I has the ability to influence the activation process of other T cells. Further analysis of naïve and effector memory CD8+ T cells is needed to understand if a similar difference is present within

this population. Studies determining the suppressive capacity of CD8+ Tcregs would be needed to choose an effective method to target the CD8+ Tcreg population further.

Finally, while we had anticipated that IFNα stimulation of T cells may be altering the cytokine milieu and contributing to the activation of T cells and the increase of TFH cells, this was not the case. However, this information is valuable as it allows us to determine that the dominant CD4+ T cell dysregulation shown in lupus like disease is likely not a direct effect of IFNα signaling.

Surprisingly, IFNα-stimulated myeloid cells played a different role in lupus like disease than we had anticipated. We had hypothesized that IFNα stimulation of myeloid cells was directly exacerbating disease, but the B6.Nba2.MyΔIFNAR mice showed a partial exacerbation in lupus-like disease presentations. Particularly evident in the B cell compartment, the inability of myeloid cells to respond to IFN-I led to increased populations of GC B cells, Memory B cells, and PCs, but a reduction in follicular B cells. The increase in the GC-associated B cell populations was paired with a reduction of neutrophils, monocytes, and MZ macrophages in the spleen. Considering the MZ macrophages are typically responsible for clearing of dead cells in the MZ/perifollicular region, it is reasonable to hypothesize that the reduction of MZ macrophages leads to an accumulation of apoptotic cells in an area that leaves them spatially poised to be a source of auto-antigen.

In addition, the decrease of monocytes and neutrophils combined with expanded populations of B and T cells known to be associated with increased disease suggested to us that the reduction of splenic myeloid cells we observed may actually be a reduction in myeloid-derived suppressor cells. This would make sense considering that the

B6.Nba2.MyΔIFNAR mice seemed to present with increases in features commonly seen elevated in disease progression, including increased populations of GC-associated B cells populations and increased activated T cells. The combination of all the data described here, in tandem with published literature, led us to generate the following speculative model (Figure 29).

Working Model Incorporating Experimental Findings

IFNα is necessary for driving B6.Nba2 lupus-like disease. The pDCs are the main IFNα producers in this model of disease. In addition, pDCs are also known to secrete TNFα and IL-6 [59,61]. TNFα works to mature the pDC population [59], while IFNα and IL-6 jointly work to develop the B cell population and increase immunoglobulin production

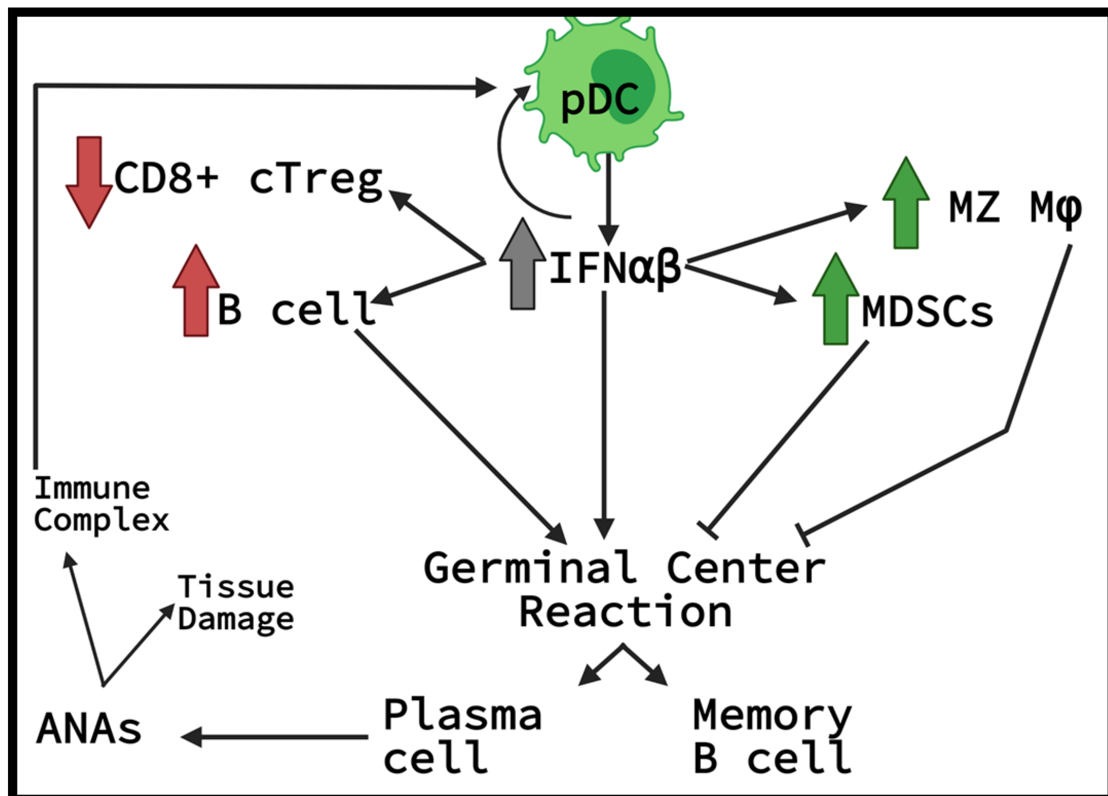

Figure 29: Model showing the effect of IFNα stimulation on immune cell subsets, as described in text.

[60]. The elevated IFNα levels in early disease trigger the activation of B cells and aid the GC B cells in avoiding apoptosis. The prolonged GC B cell survival leads to increased spontaneous GCs, and populations of Memory B and PCs.

While direct IFNα stimulation on myeloid cells increases the anti-inflammatory populations of MZ macrophages and MDSCs, which can clear dead cells from the perifollicular region of the MZ and suppress the immune response respectively, B6.Nba2 mice still present with a pro-inflammatory disease state. This is likely due to the direct effects of IFN-I on T and B cells as described above. Briefly, elevated IFNα levels directly stimulate T cells restricting the growth of CD8+ Tregs and thus allowing for the activation of T effector cells. Likewise, direct IFNα stimulation of B cells leads to an increased population of PCs and increased serum ANAs, especially those of the IgG_{2C} subtype, which can drive disease once the IFNα secretion of early disease has subsided.

Finally, the autoantibodies produced by the PCs feed-forward when they form immune complexes and drive the pDCs to continue the production of inflammatory cytokines. Since pDCs are able to respond to IFNα in an autocrine manner, the perpetuation of disease can continue, even after the disease has progressed from the IFNα initiation stage.

Discussion

One of the biggest barriers to progress in the field of SLE research and patient treatment is the extreme heterogeneity found within the SLE patient population. Many clinicians and researchers believe that SLE is likely a collection of related diseases, and that if we could better differentiate the disease subsets, we can further research and provide better care to patients.

My research regarding IFNAR in B6.Nba2 mice, combined with other data from our lab, indicates that lupus is a cyclic disease made up of two basic phases; an initiation phase and a self-perpetuation phase. In the B6.Nba2 mice, the initiation phase seems to be driven by the pDCs. Unpublished data from my lab shows that selectively depleting pDCs halts disease progression for duration of the depletion, but only if pDCs are depleted prior to 18 weeks of age (Manuscript in preparation and [61]). If pDCs are depleted later in disease, the depletion is no longer able to arrest disease progression throughout the duration of depletion. As these experiments were done through depletion of the pDC population, we must consider that pDCs also produce IL-6 and TNF-α, in addition to IFN-I [58–60]. This is notable because in combination, IL-6 and IFN-I are able to lead to increases in the PC population and associated Ig production [60] and TNFα and IFNα can contribute to pDC maturation [59]. This initiation phase can be described as "pDC Initiated/Driven Autoimmunity". I propose that all of these effects amplify the auto-immune response to a point at which the auto-immune response is self-perpetuating and no longer requires the pDCs to drive disease.

In the second phase, disease is likely sustained via other cytokines, autoantibodies and immune complexes maintaining an inflammatory state. At this point, targeting pDCs is no longer a viable way to halt disease because disease can continue without the initial signals. Data from the Rahman lab shows that IFNγ, not IFNα, is driving nephritis in the B6.Sle1 model [223], which would fit with a phasic model. This logic would also explain why ANAs are found in ~98% of SLE patients, as ANAs are key to driving disease forward and moving to a self-perpetuation state. This model is pictured in Figure 30.

Data from my research further supports this theory, as the reductions of GC-related splenic B cell populations seen in BΔIFNAR mice compared with WT B6.Nba2 mice are no longer significant in 9 month old mice, as discussed in Chapter 3. Reduced upregulation of ISGs in and a diminished response to pDC ablation in older B6.Nba2

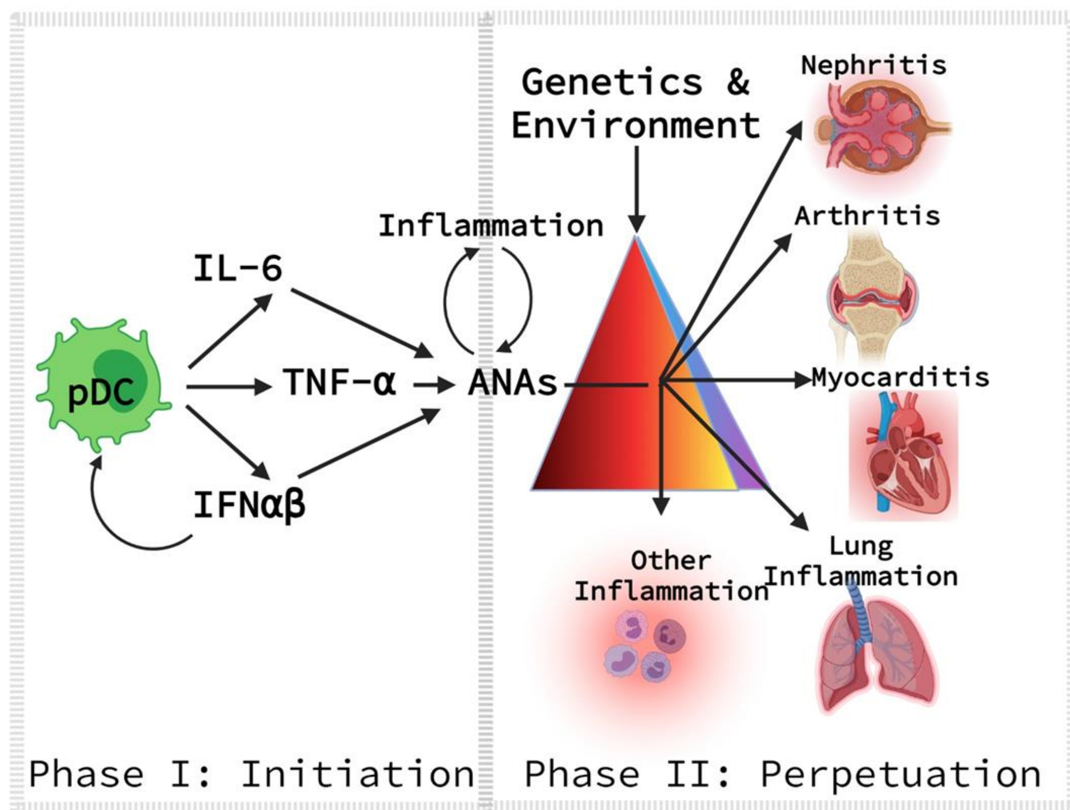

Figure 30: Model of two phase disease progression proposed in the discussion featuring an initiation phase and a perpetuation phase.

mice also supports a reduced role for IFN-I in advanced disease. It should be noted that while mouse models of lupus present with consistent disease presentations within each model, SLE in humans presents in a significantly more heterogeneous manner. This difference is likely due to the lack of genomic variety in mouse models from inbreeding, contrasting with high genomic variety among human patients. Thus, I hypothesize that the type of self-perpetuation state in human disease varies by patient and may differ based on genetic differences, exploiting existing irregularities. This differs from the two-

hit initiation hypothesis of cancer [306], in that secondary genetic irregularities are not necessary to initiate disease, but rather help determine which course and specific presentations the disease will take.

Results from the MRL/lpr IFNAR KO, where the disease becomes more severe, helps highlight that the most impactful role IFN-I is playing in lupus-like disease may in fact be increased immune cell survival [44]. In other murine models of lupus, both induced and spontaneous, the total IFNAR KO protects from disease development [19,23,28,49],. The MRL/lpr model distinctly differs from other models in that cells have an intrinsic inability to undergo apoptosis in a normal manner, due to the FAS mutation they harbor [40,41]. Thus, total IFNAR-deficiency is only able to prevent lupus-like disease if the cells are able to undergo apoptosis normally.

My data from the B6.Nba2.BΔIFNAR mice further supports this hypothesis [33]. Furthermore, the B6.Nba2.MyΔIFNAR model showed that IFNα stimulation of myeloid cells directly contributes to an increase in MZ macrophages, able to clear apoptotic cells and prevent activation of self-reactive B cells [301]. From this follows that if cells are unable to undergo apoptosis, as in the MRL/lpr model, an increased MZ macrophage population would have limited effect. Studies evaluating the effect of myeloid-specific IFNAR deficiency in MRL/lpr mice will be important to understand this process. Finally, I also found that in the absence of IFNAR expression on T cells, that lupus-prone mice were able to increase populations of splenic CD8+ Tcregs that may play a role in maintaining self tolerance.

If indeed a normal apoptotic pathway is required in addition to blocking IFN-I, this indicates that SLE patients who's disease does not respond to anifrolumab may either

have another factor driving increased pathogenic immune cell survival or their cells do not posses the ability to appropriately clear apoptotic debris. SLE patient studies report that a variety of factors related to apoptosis, cell survival, and dead cell clearance can be dysregulated in disease [307–313], futher confirming that dysregulated apoptotic pathways may need to be factored in to treatment strategies.

Impact on the field and future directions

In a disease that continues to presents heterongenously in patients and in patient response theraputics, additional theraputic targets are likely needed in order to find options that work for each subset of patients.

Throughout my studies, I have shown that the role of IFN-I in lupus is significantly more complicated than previously thought. While IFN-I stimulation of B cells can perpetuate autoantibody production and stimulation of T cells can reduce CD8+ Tcregs used to curtail autoimmunity, IFN-I stimulation of myeloid cells can promote protection. The data discussed here help shed light on why lupus responses to IFN-I may differ, in both patients and animal models. The data also highlights that timing of disease is integral in choosing the most effective path to target disease. If the cells and associated cytokines driving SLE are cycling over the course of disease, the heterogenity of pathogenesis and in response to treatment seen in patients is unsurprising, as many studies likely include patients at different stages of disease as one group.

Potential therputic targets

Importantly, the data from my research identified new pathways that could be potential theraputic targets. These are discussed further in this section and may include upstream,

downstream, or mechanistically related targets that would require validation in animal models prior to theraputic use in humans.

Survival of GC B cells

The studies in Chapter 3 relied on measuring expression of pro and anti-apoptotic factors. Prior to looking for theraputic targets, the difference in cell survival should be verified. This includes quantifying protein levels of BCL2 and $BCL_{XL,}$ likely by western blot or intracellular flow cytometry on sorted cells. IFN-inducible genes *Ifi202* and *Aim2* , implicated in survival and apoptosis, should also be evaluated, as they are encoded by the *Nba2* locus [314,315] that drives disease in the B6.Nba2 mice. Based on published literature, discussed in the following sections, surface expression of PD-1, IFIT2 and IFIT3 may also need to be quantified using the same methods.

Ex vivo, apoptosis should be evaluated by quantifying apoptotic markers, ideally cleaved caspase-3 and a combination of propidium iodide (PI) and annexin V, in sorted GC B cells and PCs. In addition, a TUNEL assay or staining for cleaved caspase-3, in combination with markers to identify the GCs and MZ, such as GL-7, SignR1, and B220, should be done on spleen sections to identify apoptotic cells in the follicles and GCs. Based on the results from Figure 15 in chapter 3, we would expect to see increased apoptosis in the germinal centers of the B6.Nba2.BΔIFNAR mice. Alternatively, if there is no visible difference in apoptotic cells in the GCs, the survival advantage confered by IFNα may be present before the GC B cell stage. This alternative hypothesis could then be tested by evaluating survival and survival/apoptotic transcripts in pre-GC B cell subsets, using methods described above or those used in Chapter 3.

One attractive theraputic option would be to reduce the prolonged survival seen in GC B cells. One of the markers expressed by the GC B cells is PD-1, and engagment of this ligand has been shown to promote long-lived antibody producing cells [316]. Of note PD-1 is expressed on other cells such as activated B cells and exhausted T cells, that are also shown to contribute to increasing lupus disease [316]. There are a variety of small molecule inhibitors that target the PD-1/PD-L1 pathway(reviewed in [317]), that could be considered for use in SLE to reduced the survival of GC B cells.

Another way to reduce survival of GC B cells would be to target BCL2. BCL2 is also regulated via phosphorylation at the post transcriptional level (reviewed in [318]). Agents blocking BCL2 have been researched in leukemias and lymphomas, so utilizing FDA approved treatment options from these diseases to treat SLE patients is an attractive prospect. There are two main drugs targeting BCL2. Navitoclax, a theraputic targeting BCL2, in addition to BCL_{XL} and $BCL_{W,}$ was never approved after early theraputic trials in CLL found that navitoclax induces presentation of thrombocytopenia in patients [319]. It is therfore unlikely that navitoclax will be successful in treating SLE. In contrast, Venetoclax was approved for treatment of chronic lymphocytic lukemia (CLL) and in specific circumstances, for acute lukemia [320]. Studies have shown that venetoclax particularly targets B cells, T cells, and IFNα producing pDCs [321,322], which indicates it may be succesful in treating SLE. Additionally venetoclax has been successfully utilized in CLL in combination with rituximab [323], which is already utilized off label in SLE,. Thus, a combination therapy may also be successful in SLE.

In addition to targeting BCL2, there are additional ways researchers may be able to target the increased cell survival. Interestingly IFIT2, also known as ISG54 and discussed

earlier in Chapter 3, is able to induce apoptosis [324]. Pro-apoptotic factors Bak and Bax are required for IFIT2-induced apoptosis [324], and thus lupus models overexpressing Bax, Bak and/or ISG54 could be utilized to see if apoptosis can be increased and disease prevented.

IFIT3 (ISG60) is able to act as a negative regulator of IFIT2-induced apoptosis [325]. Furthermore, ISG60 is found to act on IFIT2 by binding to the first tetratricopeptide repeat (TPR) and IFIT2 lacking this TPR is able to avoid negative regulation by ISG60 [324]. It may be possible to target this interaction in several ways, including but not limited to; creating a monoclonal Ab to bind ISG60 where it would otherwise bind the IFIT2 TPR, creating an inhibitor that binds near the TRP and prevents binding of ISG 60, using shRNA to suppress ISG60 expression, or using CRISPR technology to specifically alter the IFIT2 TPR so ISG60 can not bind. It has also been shown that the downstream apoptotic pathway of IFIT2 induced apoptois is via caspase 3 and mitochondrial cell death [324]. Thus, so targetting other parts of the pathway to activate the same downstram caspase-3-mediated cell death represents an alternative, alhtough less specific, approach.

Furthermore, theraputic targetting of the p53 pathway may be an additional treatment route, if IFN-inducible genes *Aim2* and *Ifi202* are shown to be altered. *Ifi202,* altered in the B6.Nba2 model, leads to the production of protein *P202* [314]. Both *P202* and *AIM2,* a fellow p200 family member [326] , have been shown to prevent p53 mediated apoptosis [314,315]. Moreover, Aim2-/- mice showed increased cell death compared to Aim2-/- Ifnar2-/- double knockout mice [315] further bolstering the potential for targetting p200 family members, like Aim2 and P202, and the cell death pathways they impair.

Myeloid derived suppressor cells

Another approach would be to increase the activity or populations of MDSCs and/or MZ macrophages. MDSCs are currently a more lofty target, as researchers are still searching for ways to identify the surface expression or intracellular markers that can distinguish them from other myeloid cells. However, as we learn more about MDSCs they will likely become a more attractive theraputic target. Prior to theraputic targetting, MDSC suppressive ability should be quantified *ex vivo*. FACs sorted GR1+ cells from B6.Nba2 and B6.Nba2.MyΔIFNAR mice should be cultured with naïve T cells and a TFH inducing cytokine cocktail. Literature has already shown that IFNα can increase the suppresive ability of Gr1+ cells [175,327], however this experiment will help determine if the decreased populations of splenic monocytes and neutrophils are indeed MDSCs. If so, we would expect to see an increase in TFH differentiation in the naïve T cells cultured with Gr1+ cells from MyΔIFNAR mice, as opposed to the naïve T cells cultured with Gr1+ cells from B6.Nba2 animals.

Luckily, more is currently known about the MZ macrophages. MZ macrophage function should be evaluated in the models discussed in Chapter 5 prior to theraputic targetting. MZ macrophage function can be quantified by using an apoptotic clearance assay, such as a sheep red blood cell (RBCs) assay, and staining spleen sections for MZ macrophages and dead cells. We would anticipate seeing a reduction in the MZ macrophage population and an increase in apoptotic cell debris in spleen sections from the MyΔIFNAR strain as compared to WT B6.Nba2 mice. Non-autoimmune B6 mice should be included as negative controls.

Expression of the scavenger receptor SignR1 is essential for apoptotic clearance by MZ macrophages [328]. Other scavenger receptors such as SCARF1, expressed on a population of CD8+ MZ DCs, have been shown to be necessary for apoptotic clearance and tolerence, with SCARF1 knockouts developing lupus-like disease [329,330]. This data suggests that future theraputics designed to upregulate scavenger receptors on MZ cell populations, such as MZ macrophages and CD8+ MZ DCs, could be extremely succesful in controlling lupus.

T regulatory Cells

Another potential target for SLE treatment would be to increase CD8+ Tcregs or modulate the signalling axis that the CD8+ Tcregs utilize to neutrilize and kill autoreactive cells. CD8+ Tcregs can utilize several difference pathways including CTLA-4 [331–333], FAS [334], and LAG-3[335–337] to neutrilize autoreactive cells. These pathways are also targeted in treatment of cancer. Many theraputic options have been developed to target these pathways and below I will discuss some of the treatments that have been approved.

Ipilimumab, an Anti-CTLA-4 IgG1 monoclonal antibody, is approved for use in high grade melanomas, however early clinical studies cited increased incidence of autoimmune responses when utilized [338], so it may not be successful in SLE. Often therapies targeting CTLA-4 are used in combination with those targeting PD-1. PD-1 is expressed on activated B and T cells (both CD4+ and CD8+), and has been shown to be more involved in reducing immune resposes in the later stage [339,340]. Nivolumab and Pembrolizumab are two theraputics that target PD-1 and both have been approved for multiple cancers. Theraputics targeting the FAS/FAS L axis have had less success in

trials, with theraputics found to induce lethal hepatitis [341]. Finally, more recent studies targetting LAG-3 show promise. LAG-3 has been found to interact synergystically with PD-1 to enable the inhibition of immune responses [342]. While clinical trials are still ongoing for LAG-3 targetting theraputics [343–345], literature indicates that targetting PD-1/PD-L1, LAG-3, or the two in combination may be most effective in reducing the immune response. Furthermore, LAG-3 knockouts have been shown to promote auto-immune diabetes [346], which indicates that therapies designed to increase cell death by upregulating LAG-3 may be successful in autoimmune diseases like SLE.

While my research was not able to define specific subsets of SLE patients based on IFNα-stimulated symptom presentation, it did provide novel insight to the field, highlight the importance of the interplay of IFNα and cell survival and clearence in disease, and provide a variety of specific targets for both existing and future theraputics to manage SLE. My research also shows that there is significant interplay between dysregulated cytokines and different cell subsets in SLE, further reinforcing the need for understanding how disease develops in different patient subsets in order to ultimately provide targeted theraputics.

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